A study of the intracellular signalling events involved in the *zona pellucida*-induced acrosome reaction in human spermatozoa

by

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DECLARATION

I, the undersigned, hereby declare that the work in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: Date:

ABSTRACT

In this study the author presents new data that will shed light on the fairly nebulous knowledge of intracellular pathways involved in the physiologically induced acrosome reaction and the subsequent events leading to fertilization. The *zona pellucida*-induced acrosome reaction, sperm-*zona* interaction as well as various sperm motion characteristics were investigated.

The first part of the study focussed on the role of extracellular signal regulated kinase (ERK), a member of the family of mitogen activated protein kinases, during the *zona pellucida*-induced acrosome reaction and sperm-oocyte interaction. It was shown that the inhibition of ERK significantly reduced the *zona pellucida*-induced acrosome reaction as measured by fluorescent microscopy. This suggests that ERKs are directly or indirectly involved in the signal transduction pathway through which the human sperm acrosome reaction is induced by the *zona pellucida*. In an attempt to provide further proof that ERK was involved in human acrosome signalling hemi*zona* assays were employed to test sperm-oocyte binding. From these sperm-oocyte-binding experiments it was clear that the inhibition of ERK leads to increased binding. These results support the idea that the *zona pellucida*-induced acrosome reaction, and therefore the physiologically relevant exocytotic event, is regulated by an ERK-mediated signal transduction process.

In the second part of the study the significance of phosphatidylinositol 3-kinase (PI3K) in the process of human sperm motility, acrosome reaction and sperm-oocyte binding, was investigated by employing the specific PI3K, LY294002. PI3K inhibition increased the percentage motility and percentage progressive motility in

asthenozoospermia patients. Under the present laboratory conditions PI3K inhibition furthermore did not influence the acrosome reaction, whilst it enhanced sperm-oocyte binding. These results therefore imply that PI3K negatively affect sperm motility and *zona*-binding.

In the last part of the study the possible effects of intracellular cGMP accumulation via acute *in vivo* sildenafil citrate (Viagra™) administration was investigated on seminal parameters, induction of the acrosome reaction, sperm-oocyte binding and sperm motility. As was expected no changes in the macro- and microscopically seminal parameters were caused by sildenafil citrate when compared to placebo. Furthermore the acrosome reaction was also not initiated or potentiated by sildenafil citrate at concentrations of 50mg orally. Sperm-oocyte binding, smooth path velocity, straight line velocity and the percentage rapid cells all increased after sildenafil citrate treatment.

From these results it appear that there are various role players in the *zona pellucida*-induced acrosome reaction intracellular signalling system. A thorough understanding of such signal transduction systems and the crosstalk in-between will ultimately yield information regarding the nature of receptors to which these signal transduction pathways are coupled in human spermatozoa as well as the intracellular effectors that ultimately regulate sperm function. Moreover, an understanding of these regulatory pathways will be essential for the future development of clinical approaches designed to enhance or preclude fertilization.

OPSOMMING

Die outeur lê in hierdie studie nuwe data voor ten einde meer lig te werp op die relatief vae veld van intrasellulêre seintransduksie paaie betrokke by die fisiologies-geïnduseerde akrosoomreaksie en die daaropvolgende gebeure wat aanleiding gee tot bevrugting. Die *zona pellucida*-geïnduseerde akrosoomreaksie, sperm-*zona* interaksie sowel as spermmotiliteitseienskappe is ondersoek.

Die eerste gedeelte van die studie fokus op die rol van ekstrasellulêre-seingereguleerde-kinase (ERK), 'n lid van die familie van mitogeen-geaktiveerde proteïenkinases, tydens die *zona pellucida*-geïnduseerde akrosoomreaksie en sperm-oösiet interaksie. Daar word aangetoon dat die inhibisie van ERK die *zona pellucida* geïnduseerde akrosoomreaksie, soos gemeet met behulp van fluorosensie mikroskopie, betekenisvol verminder. Dit suggereer dat ERK direk of indirek betrokke is by die seintransduksie paaie waardeur die akrosoomreaksie van die menslike sperm deur die *zona pellucida* geïnduseer word. In 'n poging om onomwonde te bewys dat ERK betrokke is by menslike akrosoom-seintransduksie, is hemi*zona* essais gebruik om sperm-oösiet binding te bepaal. Van hierdie sperm-oösiet binding-eksperimente is dit duidelik dat die inhibisie van ERK aanleiding gee tot verhoogde binding. Hierdie resultate ondersteun dus die idee dat die *zona pellucida*-geïnduseerde akrosoomreaksie en dus die fisiologies relevante eksositotiese gebeurtenis gereguleer word deur 'n ERK-gemediëerde seintransduksie proses.

In die tweede gedeelte van die studie is die belang van fosfatidielinositol 3-kinase (PI3K) in die proses van menslike spermmotiliteit, akrosoomreaksie en sperm-oösiet binding ondersoek deur van die spesifieke PI3K inhibitor LY294002, gebruik te maak.

PI3K-inhibisie het die persentasie motiliteit en progressiewe motiliteit by astenozoospermiese pasiënte verhoog. Onder hierdie laboratoriumtoestande het PI3K-inhibisie nie die akrosoomreaksie beïnvloed nie, terwyl sperm-oösiet binding verhoog is. Hierdie resultate beteken dus dat PI3K spermmotiliteit en *zona*-binding negatief beïnvloed.

In die laaste gedeelte van die studie is die effekte van intrasllulêre cGMP akkumulasie deur akute *in vivo* sildenafil sitraat (Viagra™) toediening op seminale parameters, induksie van die akrosoomreaksie, sperm-oösiet binding en spermmotiliteit ondersoek. Soos verwag is geen veranderinge in die makro- en mikroskopiese seminale parameters deur sildenafil sitraat in vergelyking met plasebo veroorsaak nie. Verder is die akrosoomreaksies ook nie deur 50mg orale sildenafil sitraat geïnisieer of potensieer nie. Sperm-oösiet binding, geplaneerde snelheid, reguitlyn snelheid en persentasie vinnigbewegende selle was almal vehoog na sildenafil sitraat behandeling.

Uit hierdie resultate blyk dit dat daar verskeie rolspelers in die *zona pellucida*-geïnduseerde akrosoomreaksie is. 'n Deeglike insig van al hierdie seintransduksie-paaie en die onderlinge kruiskontak tussen mekaar sal uiteindelik die nodige inligting rakende die reseptore waaraan hierdie seintransduksie paaie gekoppel is, verskaf sowel as die intrasellulêre effektore wat uiteindelik spermfunksie beheer. Nog te meer sal die begrip van hierdie regulatoriese paaie verder noodsaaklik wees vir die toekomstige ontwikkeling van kliniese benaderings om bevrugting te bevorder of te voorkom.

This dissertation is dedicated to

Wendy and Christopher

Without your tremendous patience, support and love

I would not have been able to

successfully complete

this study.

"The art of love ... is largely the art of persistence."

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ALPHABETICAL LIST OF ABBREVIATIONS

AA = Arachidonic acid

AC = Adenylate cyclase

AKAPs = A kinase-anchoring proteins

ALH = Amplitude of lateral amplitude

AR = Acrosome reaction

8-Br-cGMP = 8-bromo-cGMP

BSA = Bovine serum albumin

BCF = Beat-cross frequency

$[Ca^{2+}]_I$ = Intracellular free ionized calcium concentration

cAMP = cyclic 3',5' adenosine monophosphate

CASA = Computer assisted semen analysis

cGMP = cyclic 3',5' guanosine monophosphate

DAG = Diacylglycerol

DF = Decapacitation factors

DMSO = Dimethylsulfoxide/sucrose

ED = Erectile dysfunction

ERK = Extracellular signal-regulated kinases

FITC-PSA = Fluorescein-labeled *Pisum Sativum* agglutinin

FPP = Fertilization-promoting peptide

$GABA_A$ = γ-aminobutyric acid A

gluNAc = N-acetyl-a-D-glucosamine

G-protein = Guanine nucleotide binding protein

GTP = Guanosine triphosphate

HCL = Hydrochloric acid

HCO_3^-	= Bicarbonate
HTF	= Human tubal fluid medium
HZA	= Hemi*zona* assay
HZI	= Hemi*zona* Index
ICSI	= Intracytoplasmic sperm injection
IP_3	= Inositol-1, 4, 5-triphosphate
IVF	= *In vitro* fertilization
LC	= Lyso-phosphatidylcholine
LIN	= Linearity
LVA	= low voltage activated
MAP	= Microtubule-associated proteins
MAPK	= Mitogen-activated protein kinases
MEK	= ERK kinase
NANC	= Non-adrenergic non-cholinergic
NaOH	= Sodium hydroxide
NO	= Nitric oxide
P	= Progesterone
PA	= Phosphatic acid
PBS	= Phosphate buffered saline
PDEs	= Phosphodiesterases
PDE5	= Phosphodiesterase type 5
PI3-K	= Phosphatidylinositol 3-kinase
PIP_2	= Phosphatidyl-inositol biphosphate
PL	= Phospholipids
PLA2	= Phospholipase A2

PLC	= Phospholipase C
PLD	= Phospholipase D
PKA	= Protein kinase A
PKC	= Protein kinase C
PKG	= Protein kinase G
PtdIns	= Phosphatidylinositol
PTK	= Protein tyrosine kinases
ras-MEK-MAPK	= Mitogen-activated protein kinase
ROS	= Reactive oxygen species
SNARE	= Soluble N-ethylmaleimide-sensitive attachment protein receptors
STR	= Straightness
TK	= Tyrosine kinase(s)
TKR	= Tyrosine kinase receptor
VAP	= Average path velocity
VCL	= Curvilinear velocity (Track speed)
VOCC	= Voltage-operated calcium channels
VOCCT	= T-type voltage-operated calcium channels
VSL	= Straight-line (progressive) velocity
WHO	= World Health Organisation
ZP	= *Zona pellucida*
ZP3	= *Zona pellucida* glycoprotein 3
ZRK	= *Zona* receptor kinase

"The great tragedy of science …

the slaying of a beautiful hypothesis by an ugly fact."

- Thomas Huxley -

CHAPTER 1

INTRODUCTION AND STATEMENT OF PROBLEM

1.1 Introduction

The development of the fertilization competent state of the spermatozoon occurs through a series of poorly understood processes. Successful fertilization involves several steps including (1) movement/transit from the site of ejaculation to the site of fertilization; (2) sperm capacitation in the female genital tract; (3) binding of capacitated spermatozoa to the oocyte's extracellular coat, the *zona pellucida* (ZP); (4) induction of the acrosome reaction; (5) penetration of the ZP; and (6) fusion of the spermatozoon with the egg vitelline membrane.

Sperm-egg interaction is a carbohydrate-mediated species-specific event that initiates a signal transduction cascade resulting in the exocytosis of sperm acrosomal contents (i.e. the acrosome reaction). This step is believed to be a prerequisite that enables the acrosome reacted spermatozoa to penetrate the ZP and fertilize the egg. Researchers only recently started to investigate the intracellular mechanisms resulting in acrosomal exocytosis (i.e. fusion and vesiculation of the sperm plasma membrane and outer acrosomal membrane, allowing the exposure and release of the acrosomal contents). Although various studies have been published, very little work has been done on the intracellular signalling pathways elicited by the physiologically-induced, i.e. the *zona pellucida*-induced, acrosome reaction as most researchers make use of ligands mimicking *zona pellucida* action, thus ultimately leading to conflicting results.

1.2 Objectives and statement of the problem

Against this introductory perspective the overall objective of this research study is to present additional data that will assist reproductive biologists and clinicians in the ongoing quest to unravel the intracellular pathways involved in the physiologically induced acrosome reaction. The scarcity of human *zonae pellucidae* has placed the investigation into the physiological acrosome reaction out of reach of most reproductive biologists. Due to our fortunate accessibility to human *zonae pellucidae* we could subsequently persue investigations into this reaction.

The aim of this study was to investigate different members and mechanisms by which various intracellular signalling systems are activated during the *zona pellucida*-induced acrosome reaction and their specific roles in the subsequent events leading to fertilization. Sperm-*zona* interaction as well as various sperm motion characteristics was also investigated. All these studies were performed on human spermatozoa by making use of different signal transduction inhibitors.

1.3 Plan of study

This research project includes aspects that relate to intracellular signal transduction processes involved in human sperm function such as inducibility of the acrosome reaction, sperm-oocyte binding and motility.

As a background to the study, a broad overview of the current literature on capacitation, acrosome reaction and kinematics of human spermatozoa is provided in chapter two. Following this the basic materials used and methods followed during the research project are outlined in Chapter 3.

The experiments have been divided into four research papers that are presented as separate chapters. Each article has a specific summary, introduction, material and methods, results, discussion and reference section pertaining to the study concerned. Although each of these chapters is a complete and separate experiment, it is important to remember that the data remain closely related, since all the results have a mutual goal i.e. to further our understanding of the human sperm function in the presence of *zona pellucida*.

The central theme of Chapters 4 and 5 will focus on the role of extracellular signal-regulated kinase activation in human spermatozoa during the *zona pellucida*-induced acrosome reaction and its involvement in sperm-*zona pellucida* binding.

Chapter 6 examines the ways by which phosphatidylinositol 3-kinase inhibition enhance sperm motility parameters, while the effects of this inhibition on the *zona pellucida*-induced acrosome reaction and sperm-oocyte binding was also addressed.

The effect of sildenafil citrate on the initiation of the *zona pellucida*-induced acrosome reaction, sperm-oocyte binding and motility of human spermatozoa is the subject of Chapter 7.

The final chapter (Chapter 8) gives a retrospective look at the study. Certain aspects of the project will be highlighted and relevant suggestions will be made.

1.4 Conclusion

A thorough understanding of signal transduction in human spermatozoa will ultimately yield information regarding the nature of receptors to which these signal transduction pathways are coupled as well as the intracellular effectors that ultimately regulate sperm function. Moreover, an understanding of these regulatory pathways will be essential for the future development of clinical approaches designed to enhance or preclude fertilization.

"Live as if your were to die tomorrow.

Learn as if you were to live forever."

- Mahatma Gandhi -

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In mammals testicular sperm are morphologically differentiated but are neither progressively motile nor able to fertilize an egg. Although the ability to move forward is acquired during epididymal maturation, sperm are fertilization incompetent until after a finite period of residence in the female reproductive tract (Visconti *et al.*, 2002). Two processes, namely capacitation and acrosome reaction (AR) are of fundamental importance in the fertilization of the oocyte by the spermatozoon. Physiologically occurring in the female genital tract, capacitation is a complex process, which renders the sperm cell capable for specific interaction with the oocyte. During capacitation, modification of membrane characteristics, enzyme activity and motility properties of spermatozoa render these cells able to penetrate oocyte investments and responsive to stimuli that induces the AR prior to fertilization. The physiological AR occurs upon interaction of the spermatozoon with the *zona pellucida* (ZP) and specifically *zona pellucida* protein 3 (ZP3). This is followed by liberation of several acrosomal enzymes and other constituents that facilitate penetration of the *zona* and expose molecules on the sperm equatorial segment that allows fusion of the sperm membrane with the oolemma (Baldi *et al.*, 2000). The molecular mechanisms and the signal transduction pathways mediating the processes of capacitation and AR have been partially defined, and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodelling in the sperm's plasma membrane as well as changes in protein phosphorylation. Some of the kinases and phosphorylated proteins that are involved in the processes of

capacitation and AR have been characterised, while characterisation of sperm receptors to physiological inducers of the AR is in progress. The main signal transduction pathways involved in capacitation and AR will subsequently be summarised, as well as the various motility parameters and factors that might influence it.

2.2 Capacitation

The process of capacitation consists of a series of functional biochemical and biophysical modifications that render the ejaculated spermatozoa competent for fertilization of the oocyte. This fundamental process normally takes place in the female genital tract during the migration of spermatozoa to the site of fertilization as a consequence of specific interactions between sperm and epithelial tubal cells (Yanagimachi, 1994). However, under appropriate conditions, capacitation can also be induced *in vitro* (Yanagimachi, 1994). Most of our knowledge regarding this process has in fact been derived from *in vitro* studies. Following ejaculation, the sperm surface is surrounded by molecules, known as decapacitation factors (DF), that, until released, keeps the sperm in a non-capacitated state. These factors associate with spermatozoa following contact with seminal fluids and are progressively released from the sperm surface during capacitation. DFs thus modulates capacitation thereby leading spermatozoa to the maximal fertilizing ability at the site of fertilization (Fraser, 1999). It has been supposed that DFs, once attached to the sperm surface, activate an intracellular Ca^{2+}-ATPase maintaining low intracellular calcium concentration ($[Ca^{2+}]_i$) (Adeoya-Osiguwa & Fraser, 1996) and when they are released from the surface $[Ca^{2+}]_i$ increases. Several molecules have been indicated as possible DFs (Yanagimachi, 1994).

It was recently demonstrated that two proteins present in seminal plasma, uteroglobin and transglutaminase inhibit sperm capacitation and motility, thus representing two possible DF candidates (Luconi *et al.*, 2000). Another sperm inhibitory factor present in seminal fluid is cholesterol, which is known to inhibit several sperm functions, including capacitation (Cross, 1996; Khorasani *et al.*, 2000). In seminal plasma there are also molecules that can stimulate the fertilizing ability of spermatozoa such as fertilization-promoting peptide (FPP). FPP is a small peptide that promotes capacitation (Funahashi *et al.*, 2000; Fraser, 1998) and inhibits spontaneous acrosome loss, thereby retaining fertilization potential of sperm until the site of fertilization is reached.

Capacitation is associated with the development of a distinct motility pattern called hyperactivation (Yanagimachi, 1994), which is characterised by pronounced flagellar movements, marked lateral excursion of the sperm head and a non linear trajectory. Whether the development of hyperactivated motility is related to the biochemical and biophysical changes occurring during the process of capacitation is still a matter of debate. It is worth noting that signal transduction mechanisms involved in the development of this special sperm motility pattern are similar to those described to occur during capacitation (see later). It has recently been shown that, during *in vitro* capacitation, modifications of mitochondrial morphology, which may be relevant for the development of the hyperactivated motility pattern, also occur (Vorup-Jensen *et al.*, 1999). An additional manifestation of sperm capacitation is the acquisition of the ability to undergo the AR in response to physiological stimuli such as ZP3 and progesterone (P). The responsiveness of spermatozoa to ZP3 (Florman, 1994), P

(Baldi *et al.*, 1998) and other stimuli of the AR increases during capacitation, assuring maximal responsiveness at the site of fertilization. Capacitation involves modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activities and modulation of expression of intracellular constituents (Yanagimachi, 1994). These events are made possible by activation of a cascade of signalling pathways (schematised in Figure 1) effected by unknown mediators during transit of sperm in the female reproductive tract or during incubation *in vitro* in defined media.

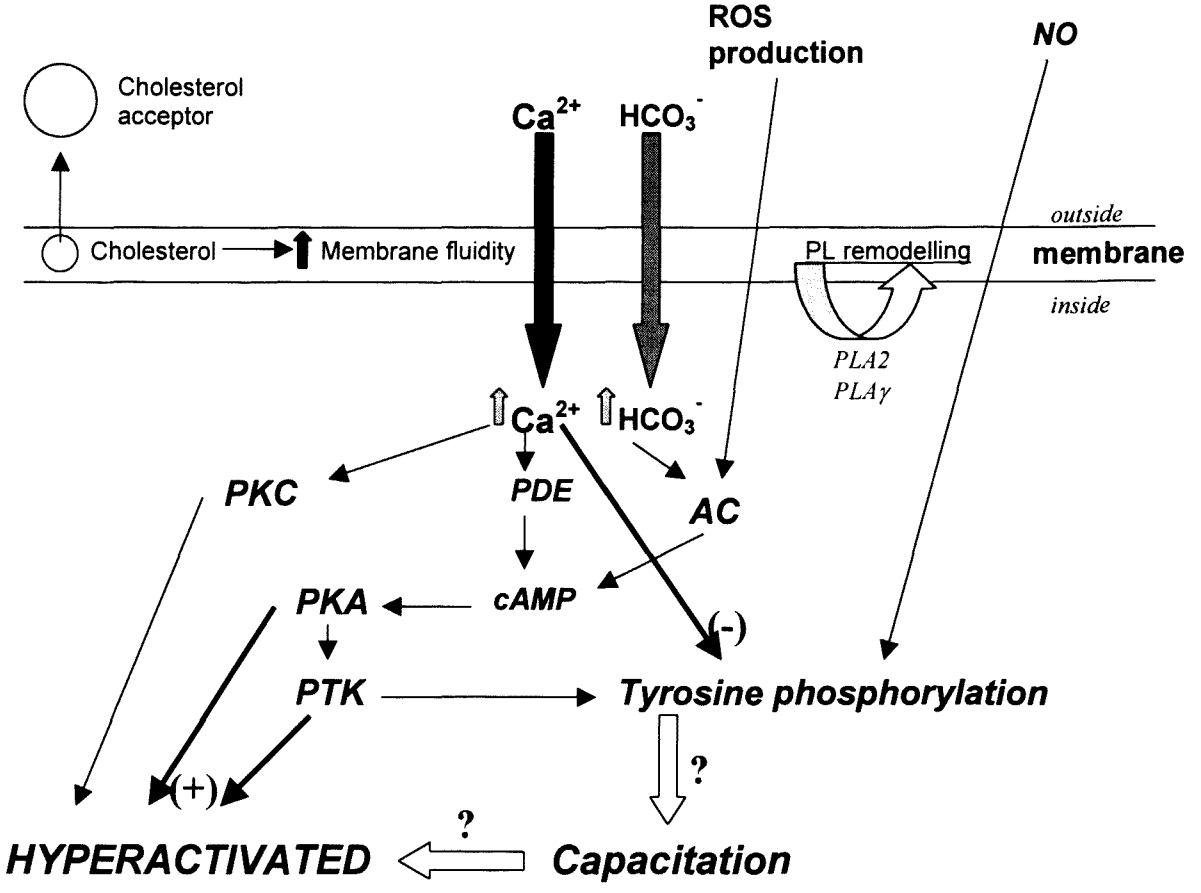

Figure 1. *Schematic representation of the main events occurring under conditions leading to capacitation and development of hyperactivated motility of human*

spermatozoa in vitro. Changes in membrane permeability to several ions have been described, among these Ca^{2+} and bicarbonate (HCO_3^-), whose influx increase during capacitation, have been reported to exert a primary role in the process. Membrane fluidity increases due to the loss of cholesterol from the membrane, which may be accelerated by the presence of a cholesterol acceptor in the external medium. Remodelling of sperm membrane phospholipids (PL) and activation of phospholipases (PLA-2 and PLCγ-1) have also been shown: in particular, increased synthesis of phosphatidylcholine from phosphatidyl-ethanolamine, phosphatidylinositol and lyso-phosphatidylcholine (LC) have been documented. A time-dependent increase of tyrosine phosphorylation of proteins is associated with development of capacitation. The increase of tyrosine phosphorylation is primarily dependent on the increase in bicarbonate, which, in turn, activates adenyl cyclase (AC) with increased generation of cAMP and subsequent activation of protein kinase A (PKA). PKA activation leads to the activation of sperm tyrosine kinase(s) (TK). On the contrary, Ca^{2+} inhibits tyrosine phosphorylation during capacitation. Other possible physiological modulators of tyrosine phosphorylation during capacitation are reactive oxygen species (ROS) that may be generated from spermatozoa or leucocytes present in the ejaculate and nitric oxide (NO). An involvement of protein kinase C (PKC) and ras-MEK-MAPK (mitogen-activated protein kinase) pathways has also been reported. (From: Baldi et al., 2000)

All these pathways may then crosstalk among each other determining the development of capacitation. However, the exact relationship among these modifications is not yet completely understood. As of yet there is no well-defined method that allows distinction of capacitated from noncapacitated spermatozoa. Capacitation does not occur synchronously in spermatozoa (Cohen-Dayag *et al.*, 1995). In addition, capacitation is transient and already capacitated spermatozoa cannot be capacitated again (Cohen-Dayag *et al.*, 1995). These complexities in *in vitro* capacitation make it difficult to appropriately interpret *in vitro* studies. To facilitate consideration of the complex cascade of molecular events that occur during capacitation, a discussion of this process may be divided into events that initiate capacitation and events that are a consequence of this process. Molecular events implicated in the initiation of capacitation include: removal of cholesterol from the sperm plasma membrane and changes in lipid distribution and composition; ion fluxes resulting in alteration of sperm membrane potential as well as concentration of intracellular calcium and other ions; and changes in protein phosphorylation and kinase activities with emphasis on an increased tyrosine phosphorylation of proteins involved in induction of hyperactivation and the AR. A working model for the initiation of capacitation based on recent work is presented in Figure 2.

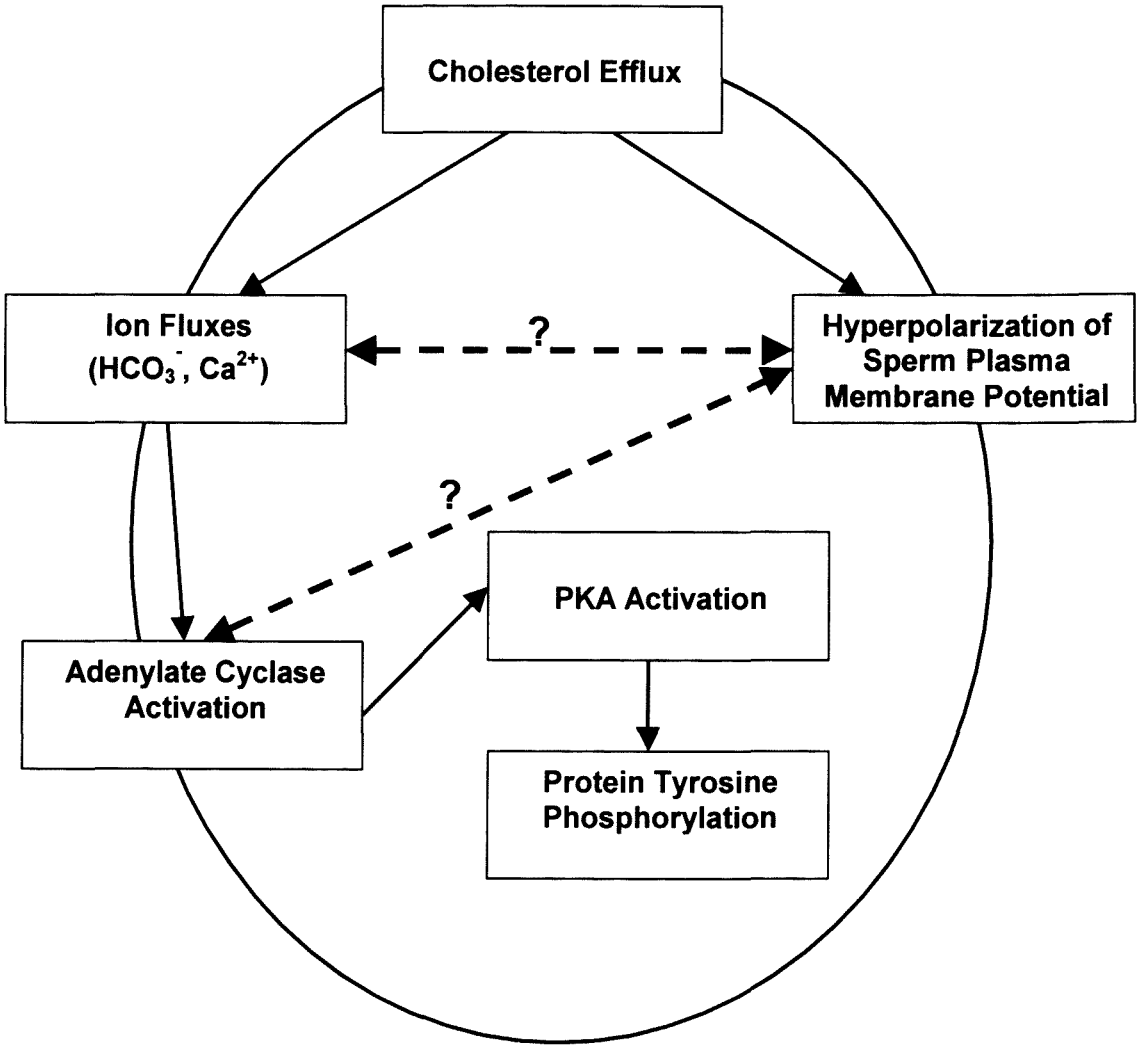

Figure 2. *Working model displaying the transmembrane and intracellular signalling pathways hypothesised to play a role in regulating sperm capacitation. This model is based on work from a number of laboratories (Visconti et al., 2002).*

2.2.1 Cholesterol efflux and changes in membrane lipids and phospholipids during capacitation

Changes in the distribution and composition of plasma membrane lipids and PL are an important feature of sperm capacitation. These changes lead to an increase in the membrane fluidity and to changes in the architecture and composition of the plasma membrane (Yanagimachi, 1994). Serum albumin, an essential component of *in vitro* capacitation media, is believed to function as a sink for cholesterol by removing it from the sperm plasma membrane (Davis *et al.*, 1979; Davis *et al.*, 1980; Davis, 1981; Go & Wolf 1985; Langlais & Roberts, 1985; Suzuki & Yanagimachi, 1989; Cross, 1996; Cross, 1998). Although serum albumin may have other roles during capacitation (Espinosa *et al.*, 2000), its ability to facilitate cholesterol efflux is required for capacitation. For example, capacitation is inhibited by the addition of cholesterol and/or cholesterol analogues to the capacitation medium (Visconti *et al.*, 1999b). Furthermore, serum albumin can be substituted in *in vitro* capacitation media with cholesterol-binding compounds such as high density lipoproteins (HDL) (Therien *et al.*, 1997; Visconti *et al.*, 1999b) and cyclodextrins (Choi & Toyoda, 1998; Cross, 1999; Osheroff *et al.*, 1999; Visconti *et al.*, 1999a) to induce capacitation. The removal of cholesterol and likely other sterols (e.g. desmosterol) (Visconti *et al.*, 1999b) from the plasma membrane is upstream of multiple signalling events intrinsic to the capacitation process. Visconti and co-workers (1999) have demonstrated that heptasaccharides (cholesterol-binding molecules) promote the release of cholesterol from the plasma membrane of mouse sperm in the absence of bovine serum albumin (BSA), increase tyrosine phosphorylation and promote capacitation of mouse sperm as measured by the ability of the ZP to induce the AR and by successful fertilization *in vitro*. These data suggest that cholesterol release is the signal that activates

membrane signal transduction pathways related to capacitation (Visconti *et al.*, 1999).

The removal of sterols decreases the cholesterol:phospholipid molar ratio in the sperm plasma membrane as assessed by different criteria (Davis, 1981; Tesarik & Flechon, 1986; Hoshi *et al.*, 1990; Cross 1998). This could account for the membrane fluidity changes (Wolf & Cardullo, 1991; Wolf *et al.*, 1986) and redistribution of membrane proteins, observed with lectins (Cross & Overstreet, 1987) and antibodies (Shalgi *et al.*, 1990; Rochwerger & Cuasnicu, 1992) that occur during capacitation. From the standpoint of cell signalling, capacitation-associated alterations, in the transbilayer phospholipid behaviour resulting in membrane lipid disorders, were recently reported to occur through a cAMP-dependent pathway after exposure of boar sperm to HCO_3^- (Gadella & Harrison, 2000). Therefore, multiple plasma membrane modifications appear to contribute to the process of capacitation. It is also important to consider what component of the female tract fluid might serve as a cholesterol acceptor *in vivo*. Since fluids of the female tract are partially derived from serum, serum-associated sterol acceptors could function *in vivo*. The identity of such acceptors remains to be clarified.

The total amount of PL does not appear to change considerably during capacitation (Yanagimachi, 1994). However, capacitation is associated with an increase of phospholipid methylation and increased synthesis of phosphatidylcholine from phosphatidylethanolamine (Llanos & Meizel, 1983). Incubation of spermatozoa under capacitating conditions, in the presence of bicarbonate, does not alter phospholipid distribution (Harrison & Gadella, 1995). Such conditions, however, strongly inhibits

phospholipid transfer and leads to a slow increase of phosphatidylcholine concentration in the inner leaflet of the membrane (Harrison & Gadella, 1995). Recently, Gadella and Harrison (2000) have shown that the inclusion of bicarbonate in the capacitating medium increases the translocation of phosphatidylcholine and sphingomyelin from the outer to the inner leaflet of the membrane, possibly due to activation of a bi-directional translocase (scramblase). Levels of phosphatidylinositol and LC increase during capacitation *in vivo* in porcine sperm (Snyder & Clegg, 1975). In view of the fusogenic properties of lysophospholipids, an increase in their relative amount may be relevant to prepare the sperm for the AR.

The molecular basis of signalling events induced by cholesterol efflux from sperm is not well understood. In somatic cells, cholesterol removal is thought to disrupt lipid rafts activating signalling events involving tyrosine kinases (TK), guanine nucleotide binding proteins (G-proteins), and/or other molecules (Kabouridis *et al.*, 1997; Brown & London, 1998; Roy *et al.*, 1999). The activation of similar signalling events during capacitation in sperm correlates to the removal of cholesterol from the plasma membrane. In sperm, cholesterol may likewise be concentrated in specialised plasma membrane microdomains such as lipid rafts and caveolae (Brown & London, 1998). This idea is supported by the recent finding that one important component of caveolae, caveolin 1, is present in the plasma membrane overlying the acrosomal region and the flagellum of mouse and guinea pig sperm (Travis *et al.*, 2000). The hypothesis that lipid rafts and caveolae concentrate signalling complexes in the sperm plasma membrane warrants continued investigation.

2.2.2 Ion fluxes and the regulation of sperm plasma membrane potential

During transit through the male and female reproductive tracts, sperm are exposed to significant changes in the extracellular milieu, including variations in extracellular ion concentrations and osmolarity. For example, caudal epididymal sperm are stored in an environment that contains high K^+, low Na^+ and very low HCO_3^- concentrations (Brooks, 1983; Setchell *et al.*, 1994). These ion concentrations radically change when the sperm are ejaculated, first in the seminal fluid and then in the female tract, where the K^+ is significantly reduced and the Na^+ and HCO_3^- concentrations are significantly increased (Brooks, 1983; Setchell *et al.*, 1994; Yanagimachi, 1994). These dramatic shifts in extracellular ion concentrations trigger modulations in intracellular ion concentrations and consequently lead to an altered membrane potential of the sperm plasma membrane.

Changes in intracellular ion concentrations have been associated with different aspects of sperm function such as sperm motility in trout sperm (Morisawa & Suzuki, 1980; Gatti *et al.*, 1990), capacitation in mammalian sperm (Visconti *et al.*, 1995a; Zeng *et al.*, 1995; Arnoult *et al.*, 1999) and the acrosome reaction in sperm from multiple species (Arnoult *et al.*, 1996; Darszon *et al.*, 1999). The dramatic influence of the external ion composition and the effect of channel blockers on sperm motility, capacitation, and the AR strongly suggest that ion channels actively participate in the regulation of sperm function. Ion channels can catalyse the flow of millions of ions through the non-conducting lipid bilayer; therefore, a few ion channels can cause changes in a small cell like the sperm within milliseconds (Darszon et al., 1999). Ion concentrations determine the plasma membrane potential through ion-selective channels and control the extent of channel activity and ion flow. The plasma

membrane potential can also regulate ion channel activity as well as second messenger levels. For example, in trout sperm, changes in the plasma membrane potential by changes in extracellular K^+ concentration modulate sperm motility through a cAMP pathway (Morisawa & Ishida, 1987). Moreover, Beltran *et al.*, (1996), demonstrated in sea urchin sperm that cAMP synthesis could be regulated by changes in membrane potential.

In vitro, the resting membrane potential is determined by the relative permeabilities of the sperm plasma membrane for ions that constitute the capacitation media. Under normal conditions, sperm maintain an internal ion concentration markedly different from that in the extracellular medium and these differences establish the resting plasma membrane potential. The ion composition of capacitation media mimics that of oviductal fluid (Yanagimachi, 1994). These media are high in Na^+ (about 130 mM) and Cl^- (about 100 mM), but low in K^+ (about 5.9 mM). Capacitation media also contain Ca^{2+} (about 2.7 mM) and HCO_3^- (10-25 mM). In contrast, intracellular fluids of sperm have a low concentration of Na^+ (about 14 mM) and high concentration of K^+ (about 90-120 mM) (Babcock, 1983; Zeng *et al.*, 1995). The free intracellular Ca^{2+} concentration of non-capacitated sperm is approximately 0.1 M or less, but during the AR it may increase to approximately 10 M (Bailey & Storey, 1994; Arnoult *et al.*, 1999). To date, the intracellular concentrations of Cl^- and HCO_3^- in sperm have not been determined. These differences between extracellular and intracellular ion concentrations are established by the respective ion permeabilities and determine the resting membrane potential in mammalian sperm.

Recently, Zeng *et al.*, (1995) demonstrated that capacitation is accompanied by hyperpolarization of the sperm plasma membrane. Membrane hyperpolarization may be partially due to an enhanced K^+ permeability related to the decrease in inhibitory modulation of K^+ channels during capacitation (Zeng *et al.*, 1995). Since capacitation prepares the sperm for the AR, capacitation-associated hyperpolarization may regulate the ability of sperm to generate transient Ca^{2+} elevations during AR by physiological agonists (e.g. ZP of the egg or P). This hypothesis is consistent with the presence of low voltage activated (LVA) Ca^{2+} T-channels in spermatogenic cells (Arnoult *et al.*, 1996; Lievano *et al.*, 1996) that may also be present in mature sperm. A signature property of LVA Ca^{2+} channels is a low threshold for voltage-dependent inactivation. These Ca^{2+} channels are inactivated at holding potentials between -80 and -60 mV and cannot be activated readily from more positive holding potentials (Arnoult *et al.*, 1996; Lievano *et al.*, 1996). Thus, if LVA Ca^{2+} T-channels are involved in the regulation of the AR, sperm must maintain a hyperpolarized membrane potential during the early stages of interaction with the egg (Arnoult *et al.*, 1999; Florman *et al.*, 1998). Presently, little is known about the regulation of capacitation-associated hyperpolarization. In order to understand the ionic basis of these changes in sperm plasma membrane potential, it will be necessary to analyse how the aforementioned ion permeabilities change during capacitation.

2.2.2.1 Modification in concentration of intracellular calcium and other ions during capacitation (HCO_3^-, Ca^{2+}, and the cAMP pathway)

Numerous studies have demonstrated that capacitation is Ca^{2+}-dependent (DasGupta *et al.*, 1993; Visconti *et al.*, 1995a). The initiation and/or regulation of capacitation by Ca^{2+} occur via different targets, some of which are involved with

cAMP metabolism. Since in sperm Ca^{2+}/calmodulin can activate both the synthesis of cAMP by adenylate cyclase (Gross *et al.*, 1987), as well as degradation by cAMP cyclic nucleotide phosphodiesterase (Wasco & Orr, 1984), it is not surprising that Ca^{2+} has both positive and negative actions on capacitation and related signalling events. In this respect, Ca^{2+} has a positive effect on mouse sperm by inducing capacitation-associated changes in protein tyrosine phosphorylation (Visconti *et al.*, 1995a). In contrast, Ca^{2+} has been demonstrated to inhibit protein tyrosine phosphorylation in human sperm during the first 2 h of *in vitro* capacitation (Carrera *et al.*, 1996; Luconi *et al.*, 1996). An increase in intracellular sperm Ca^{2+} during capacitation has been described by some investigators, whereas others have shown that no changes in Ca^{2+} levels occur during this maturational event (Yanagimachi, 1994). This ambiguity could be due, in part, to the well-demonstrated action of Ca^{2+} on the AR and to the inherent difficulties in differentiating between these events. However, the action of Ca^{2+} at the level of effector enzymes involved in sperm signal transduction suggests that this divalent cation is likely to play an important role in capacitation.

Modification of intracellular concentration of calcium ions ($[Ca^{2+}]_i$) is the most fully characterised biochemical event during capacitation. An increase in the concentration of Ca^{2+} during capacitation has been demonstrated in several mammalian species (Yanagimachi, 1994) including human (Baldi *et al.*, 1991; Garcia & Meizel, 1999). Extracellular Ca^{2+} is indeed one of the necessary constituents for the completion of capacitation of spermatozoa *in vitro* (Yanagimachi, 1994). In spermatozoa $[Ca^{2+}]_i$ is regulated by a Ca^{2+}-ATPase (acting as a Ca^{2+} extrusion pump) (Fraser & McDermott, 1992), Ca^{2+}/H^+ exchanger system and Na^+/Ca^{2+} antiporter (acting as Ca^{2+} entrance

systems) in the plasma membrane (Fraser, 1995), and by putative intracellular Ca^{2+} stores, whose presence in human sperm has been suggested by several evidences (Blackmore, 1992; Walensky & Sneider, 1995; Dragileva *et al.*, 1999; O'Toole *et al.*, 2000). However, the role of intracellular calcium stores in the physiology of spermatozoa is still questioned (O'Toole *et al.*, 2000; Kirkman-Brown *et al.*, 2000; Kobori *et al.*, 2000). Recent data (Dragileva *et al.*, 1999) indicate that cytosolic Ca^{2+} is actively transported into the acrosome by an ATP-dependent, thapsigargin-sensitive pump and that it may be released from the acrosome through an inositol-1,4,5-triphosphate (IP_3)-gated calcium channel. More recently, the existence of store-operated calcium channels that mediate sustained calcium increase in response to ZP3 in mouse sperm has been demonstrated (O'Toole *et al.*, 2000). The location of such stores remains to be demonstrated since endoplasmic reticulum is not present in mature spermatozoa and the acrosome does not appear (according to recent studies performed in individual spermatozoa) to retain significant amounts of calcium (Kirkman-Brown *et al.*, 2000; Kobori *et al.*, 2000). It has been hypothesised that modulation of the activity of the Ca^{2+}-extrusion system, in particular Ca^{2+}-ATPase, occurs during capacitation leading to an increase in intracellular Ca^{2+} (Fraser & McDermott, 1992; Fraser, 1995). Drugs such as quercetin, that inhibit Ca^{2+}-ATPase, accelerate capacitation (Fraser & McDermott, 1992; Fraser, 1995). A Na^{+}/Ca^{2+} exchanger is present in mammalian sperm, however, its role in controlling intracellular Ca^{2+} during capacitation is not clear. Similarly, although voltage-operated calcium channels (VOCC) have been demonstrated in mammalian spermatozoa (Benoff, 1998), their role in regulating intracellular calcium concentration during capacitation is not clear. Moreover, although the presence of T-type voltage-operated calcium channels (VOCCT) in germ cells has been unequivocally demonstrated

(Arnoult *et al.*, 1996; Darszon *et al.*, 1999) and their involvement in the effect of ZP3 on mature sperm indicated (Arnoult *et al.*, 1996, O'Toole *et al.*, 2000), the role of these channels in the process of capacitation is far from being defined. However, it seems quite clear that sperm VOCCT may be modulated during capacitation. Indeed, during this process the sperm membrane becomes hyperpolarized due to enhanced K^+ permeability (Brewis *et al.*, 2000). This hyperpolarization may act to prime VOCCT from an inactivated state to a closed one, which can be activated by an agonist inducing depolarisation such as ZP3 (Arnoult *et al.*, 1998). Downstream targets of calcium include the calcium-binding protein calmodulin, whose involvement in sperm capacitation has been recently demonstrated (Si & Olds-Clarke, 2000). Besides Ca^{2+}, intracellular K^+ (Zeng *et al.*, 1995), Na^+ (Hyne *et al.*, 1985) and Cl^- (Fraser, 1995) concentrations have been shown to be modulated during capacitation. The increase of intracellular Na^+ appears to be important for capacitation, since the Na^+ ionophore, monensin, promotes this process in mouse sperm (Fraser, 1995). The intracellular concentration in zinc ion decreases in the acrosome of hamster spermatozoa during capacitation (Andrews *et al.*, 1994). In addition, incubation of spermatozoa in a zinc-containing medium inhibits the process (Andrews *et al.*, 1994). These findings suggest that zinc may play a role in destabilisation of plasma membrane during capacitation (Andrews *et al.*, 1994).

A rise in intracellular pH has been reported during capacitation of bovine sperm (Vredenburgh-Wilberg and Parrish, 1995). However, the role of pH in sperm capacitation is not yet clear, since an artificial increase in intracellular pH in spermatozoa does not accelerate the process (Fraser, 1995). Cross *et al.* (1997) have shown that a cholesterol efflux from the plasma membrane, during the

capacitation of human spermatozoa, determines a rise in intracellular pH and responsiveness to P. Recent data have demonstrated the presence of a Na^+ dependent Cl^-/HCO_3^- exchanger that regulates bicarbonate transport and motility in mature sperm (Zeng *et al.*, 1996), however the role of this pump in capacitation is not clear. It has been shown that bicarbonate regulates adenylate cyclase (AC) activity and cAMP metabolism (Visconti *et al.*, 1990) and is necessary for tyrosine phosphorylation of proteins during capacitation (see later). It is of interest that bicarbonate concentration is low in the epididymis and high in the seminal plasma and in the oviduct indicating that modifications of bicarbonate concentration in reproductive tracts play an important role in the suppression of capacitation in the epididymis and in the promotion of this process in the female reproductive tract (Wassarman, 1999).

Numerous studies have demonstrated that capacitation is HCO_3^--dependent (Lee & Storey, 1986; Neill & Olds-Clarke, 1987; Boatman & Robbins, 1991; Shi & Roldan, 1995; Visconti *et al.*, 1995a). Little is known about the mechanisms of HCO_3^- transport in sperm. However, the ability of 4,4'-diidothiocyanatostilbene-2,2'-disulfonic acid (DIDS), a well-known inhibitor of anion transporters, to inhibit the actions of HCO_3^- on various sperm functions suggests that sperm contain functional anion transporters (Okamura *et al.*, 1988; Visconti *et al.*, 1990; Spira & Breitbart, 1992;Visconti *et al.*, 1999c).

The transmembrane movement of HCO_3^- anions into sperm could be responsible for the known increase in intracellular pH that is observed during capacitation (Uguz *et al.*, 1994; Zeng *et al.*, 1996). An additional target for the action of this anion could be

the regulation of sperm cAMP metabolism, since the synthesis of cAMP by mammalian sperm AC is markedly stimulated by HCO_3^- (Okamura *et al.*, 1985; Garty & Salomon, 1987; Visconti *et al.*, 1990). The increase in cAMP during capacitation and the stimulation of AC activity in sperm by increased levels of intracellular HCO_3^- and Ca^{2+} implicate a role for this enzyme and the cAMP-signalling pathway in capacitation. AC in sperm has been the subject of multiple studies, but whether one or more proteins represent it remains controversial.

Sperm AC has unique properties when compared with somatic cell cyclases (Garbers & Kopf, 1980; Leclerc & Kopf, 1995). For example, unlike somatic cell cyclases, responses of sperm AC to agents that modulate stimulatory GTP binding proteins (Gs), such as cholera toxin, AlF4- or GTP analogues, are weak or completely absent. Since no cholera toxin-ADP ribosylated substrate has been detected in mammalian sperm, the low sensitivity to G-protein effectors could be due to the lack of Gs protein in these cells (Hildebrandt *et al.*, 1985). Another possibility is that the sperm AC is unable to interact with Gs proteins due to differences in cyclase tertiary structure. As mentioned above, an important property of the sperm AC is its regulation by HCO_3^- anion (Okamura *et al.*, 1985).

Recent studies suggest that the sperm AC is a post-translationally modified form of the testicular soluble AC (Buck *et al.*, 1999). Similar to the sperm AC activity, the enzymatic activity of recombinant testicular soluble AC is stimulated by HCO_3^- anions (Chen *et al.*, 2000). In addition, antibodies against the catalytic domain of the testicular soluble AC recognised two sperm proteins in corresponding to the deduced molecular masses of the processed and unprocessed forms of the testicular enzyme

(Chen *et al.*, 2000) suggesting that this cyclase remains associated with sperm after spermatogenesis. Interestingly, the sequence from the catalytic domain of this cyclase has sequence homology to cyanobacterial AC and the cyanobacterial cyclase is also HCO_3^--dependent (Chen *et al.*, 2000). Although the testis cyclase has been found in the soluble fraction, it is significant that cyclase activity identified in mammalian sperm remains associated with the particulate membrane fraction. Therefore, the testicular soluble AC found would be predicted to have a mechanism allowing for translocation from the cytosol to the membrane at some point during spermatogenesis.

2.2.3 Changes in protein phosphorylation and protein kinase activity during capacitation

Protein phosphorylation during sperm capacitation has been widely studied in the last five years, leading to the generation of more than 100 papers in the international literature. The best studied kinases involved in capacitation are Ca^{2+}-calmodulin activated kinases, cAMP-dependent kinases (PKA), calcium and phospholipid activated protein kinase (PKC), which induce phosphorylation of proteins in serine and threonine residues, and TKs, which phosphorylate proteins in tyrosine residues. The involvement of kinase pathways in the process of capacitation has been mainly studied by using inhibitors of such pathways. However, specificity of kinase inhibitors is doubtful and many of them may affect other intracellular signal transduction pathways. In this light, caution should be applied in interpreting results of these studies (Baldi *et al.*, 2000).

2.2.3.1 Involvement of AC/cAMP/PKA pathway in capacitation

A spontaneous increase of cAMP during capacitation has been demonstrated (White & Aitken, 1989) and inhibitors of PKA, the serine-threonine kinase activated by this pathway, inhibit capacitation (Aitken *et al.*, 1998). Pentoxifylline, which promotes an increase in cAMP by inhibiting sperm phosphodiesterases, induces capacitation (Ain *et al.*, 1999). It has been well established that Ca^{2+} and HCO_3^- stimulate AC but the exact mechanism by which these ions activate AC is not clear (Visconti *et al.*, 1990; Garty & Salomon, 1987). cAMP generated during capacitation and subsequent activation of PKA appears to play a key role in the increase of tyrosine phosphorylation during capacitation (Visconti *et al.*, 1999, also see later). Since PKA may phosphorylate several cellular substrates, its sequestration in specific cellular compartments is necessary to spatially restrict its action, thus ensuring specificity of functions. Compartmentalisation of PKA is accomplished by A-kinase-anchoring proteins (AKAPs) which have been recently characterised in sperm of different species (Carerra *et al.*, 1996; Moss *et al.*, 1999; Vijayaraghavan *et al.*, 1999). Their specific localisation to the tail of the sperm indicates a role for these proteins in the modulation of sperm motility (Vijayaraghavan *et al.*, 1999). Recently, a role for the AC/cAMP/PKA pathway in plasma membrane lipid remodelling, occurring during capacitation, has been demonstrated (Harrison & Miller, 2000). Yet, many questions still remain unanswered concerning the AC/cAMP/PKA pathway in human sperm. Although the specific expression of a soluble AC has been shown in male germ cells (Sinclair *et al.*, 2000), the types of phosphodiesterases (PDEs) present in these cells remain to be defined. Recently, Richter *et al.* (1999) detected, the presence of mRNA transcripts for several PDEs in ejaculated human spermatozoa, but no conclusions

can be drawn concerning the type of PDE specifically expressed in mature spermatozoa.

2.2.3.2 Involvement of PKC in capacitation

The presence of PKC in mammalian spermatozoa and its role in sperm motility and the process of AR are documented (Breitbart & Noar, 1999), but PKC activity is very low compared to somatic cells (Breitbart & Noar, 1999, Bonaccorsi *et al.*, 1998). The role of this enzyme during capacitation is poorly understood. Early studies demonstrated that stimulation of PKC with phorbol esters accelerates the process of capacitation (Rotem *et al.*, 1992). This effect was inhibited by PKC inhibitors, suggesting that PKC may be involved in capacitation (Rotem *et al.*, 1992). In addition, PKC may also be involved in epidermal growth factor-induced capacitation (Furuya *et al.*, 1993). All these studies were performed using high levels of phorbol esters as PKC inducers. Although it was shown that PKC activity of human sperm can be stimulated by a phorbol ester (Bonaccorsi *et al.*, 1998), additional effects of these tumour promoters on other sperm kinases cannot be excluded. Recently, the role of PKC in capacitation has been questioned (Ain *et al.*, 1999).

2.2.3.3 Involvement of tyrosine phosphorylation in capacitation

The first evidence for the presence of tyrosine phosphorylated proteins in mammalian spermatozoa dates back to 1989 (Leyton & Saling, 1989). Using anti-phosphotyrosine antibodies, Leyton and Saling (1989) identified three different phosphoproteins at 52, 75, and 95 kDa in the mouse spermatozoa. The 95 kDa protein was tyrosine phosphorylated under all experimental conditions and including interaction of spermatozoa with solubilized ZP proteins (Leyton & Sailing, 1989).

Capacitation is characterised by a spontaneous, time-dependent increase of tyrosine phosphorylation of different proteins (Luconi *et al.*, 1995; Visconti *et al.*, 1995). The main tyrosine phosphorylated proteins are in the range of 95-100 kDa (Leyton & Saling, 1989; Luconi *et al.*, 1995; Visconti *et al.*, 1995; Luconi *et al.*, 1996; Osheroff *et al.*, 1999). Capacitation-associated changes in protein tyrosine phosphorylation have been demonstrated in multiple species including the mouse (Visconti *et al.*, 1995a), bovine (Galantino-Homer *et al.*, 1997), human (Leclerc & Kopf, 1995; Osheroff *et al.*, 1999), pig (Kalab *et al.*, 1998) and hamster (Devi *et al.*, 1999; Visconti *et al.* 1999c; Jha & Sjivaji, 2001). In the mouse, *in vitro* capacitation of caudal epididymal sperm promotes tyrosine phosphorylation of a subset of proteins between Mr 40000 and 120000 (Visconti *et al.*, 1995a). At least in the mouse, the increase of protein tyrosine phosphorylation is dependent on the presence of Ca^{2+}, $NaHCO_3^-$ and BSA (Visconti *et al.*, 1995). Specifically, the absence of any one of these media constituents prevents both protein tyrosine phosphorylation and capacitation. It is necessary to mention that the effect of media constituents on protein tyrosine phosphorylation and capacitation varies slightly from species to species (Visconti *et al.,* 1999c; Jha & Sjivaji, 2001). It is worth to note that some of these results have not been confirmed in human sperm. Indeed, in humans, Ca^{2+} and tyrosine phosphorylation seem to be inversely related and protein tyrosine phosphorylation is enhanced in calcium free medium (Luconi *et al.*, 1996; Carrera *et al.*, 1996) indicating that calcium might induce the activation of tyrosine phosphatases (Carrera *et al.*, 1996). The dependence of *in vitro* protein tyrosine phosphorylation on serum albumin, or other cholesterol acceptors, indicates a correlation between cholesterol efflux and cAMP-induced tyrosine phosphorylation. It has been demonstrated that in BSA-deprived media protein tyrosine phosphorylation and sperm capacitation are

inhibited (Visconti *et al.*, 1995; Osheroff, 1999). Whether cholesterol removal is upstream from or co-incidental with the action of Ca^{2+} and/or $NaHCO_3^-$ is not presently known. It is hypothesised that cholesterol removal, with a resultant change in sperm plasma membrane fluidity, modulates Ca^{2+} and/or HCO_3^- ion fluxes leading to AC activation; this hypothesis remains to be tested.

The increase in protein tyrosine phosphorylation is regulated by an AC/cAMP-dependent pathway that involves PKA in sperm from the mouse (Visconti *et al.*, 1995b), bull (Galantino-Homer *et al.*, 1997), human (Leclerc *et al.*, 1996; Osheroff *et al.*, 1999), boar (Kalab *et al.*, 1998) and hamster (Visconti *et al.*, 1999c), as well as by reactive oxygen species generated at the beginning of capacitation (LeClerc *et al.*, 1998; see later). In addition, recent data suggest a role for nitric oxide generated by human spermatozoa in the promotion of capacitation (Herrero *et al.*, 1998; Francavilla *et al.*, 2000) and the increase of tyrosine phosphorylation (Herrero *et al.*, 1998).

The involvement of PKA is indicated since inhibitors of PKA activity are able to inhibit tyrosine phosphorylation as well as capacitation. Since PKA is not able to phosphorylate proteins on tyrosine residues, an intermediate TK may be involved. Figure 3 summarises three possible mechanisms: (1) direct or indirect stimulation of a TK by PKA; (2) direct or indirect inhibition of a phosphotyrosine phosphatase; and (3) direct or indirect phosphorylation of proteins by PKA on serine or threonine residues that prime these proteins for subsequent phosphorylation of tyrosine residues. These distinct possibilities are currently being explored in different

laboratories. The identification of the enzymes responsible for the tyrosine phosphorylation pathway(s) will improve our knowledge of the capacitation process.

As stated above, in human sperm the highest degree of tyrosine phosphorylation was found in a protein of 95-97 kDa (Leyton & Saling, 1989; Luconi *et al.*, 1995; Visconti *et al.*, 1995; Luconi *et al.*, 1996; Osheroff, 1999). A tyrosine-phosphorylated protein in this molecular weight range was previously indicated as the possible sperm receptor for ZP3, *zona* receptor kinase (ZRK) (Burks *et al.*, 1995). This protein has been characterised, partially cloned and sequenced. Its sequence shows a 55% homology with the receptor-like PTK c-eyk (Burks *et al.*, 1995) and 97-100% homology with the proto-oncogene c-mer (Bork *et al.*, 1996). Among the other tyrosine phosphorylated proteins in the same molecular weight range, an AKAP specifically expressed at the tail level has been recently identified (Carrera *et al.*, 1996; Moss *et al.*, 1999; Vijayaraghavan *et al.*, 1999). This protein may be involved in the development of the hyperactivated motility pattern. An extracellular signal regulated kinase pair (ERK-1 and ERK-2) of sperm proteins that are phosphorylated on tyrosine and activated during sperm capacitation was also identified (Luconi *et al.*, 1998; Luconi *et al.*, 1998). Their inhibition with a pharmacological compound suppresses capacitation (Luconi *et al.*, 1998), indicating a role for these proteins in the process. Immunofluorescence labelling of phosphotyrosine residues, indicated that capacitation as well as exposure to *zona* proteins increased the degree of tyrosine phosphorylation in each spermatozoon and the number of sperm cells phosphorylated in the acrosomal region of the sperm head (Naz *et al.*, 1991). Incubation of spermatozoa with antiphosphotyrosine antibodies or inhibition of TK activity inhibited zona-free hamster egg penetration (Naz *et al.*, 1991), prevented AR

and blocked fertilization (Leyton *et al.*, 1992). However, whether tyrosine phosphorylation is fundamental for the development of the capacitated state or is simply associated to the phenomenon stay a matter of debate. It was also shown that erbstatin, a potent inhibitor of TK, did not inhibit capacitation measured as the ability of spermatozoa to respond to P (Luconi *et al.*, 1996). Similarly, Ain *et al.* (1999), have reported that thyrphostin A-47, a PTK inhibitor, does not inhibit pentoxifylline-stimulated capacitation, although it suppress the AR stimulated by this agent. To understand the role of tyrosine phosphorylation in the process of capacitation, we will probably need to wait for the characterisation of many, if not all, of the tyrosine phosphorylated proteins as well as the TKs that are activated during the process (Baldi *et al.*, 2000).

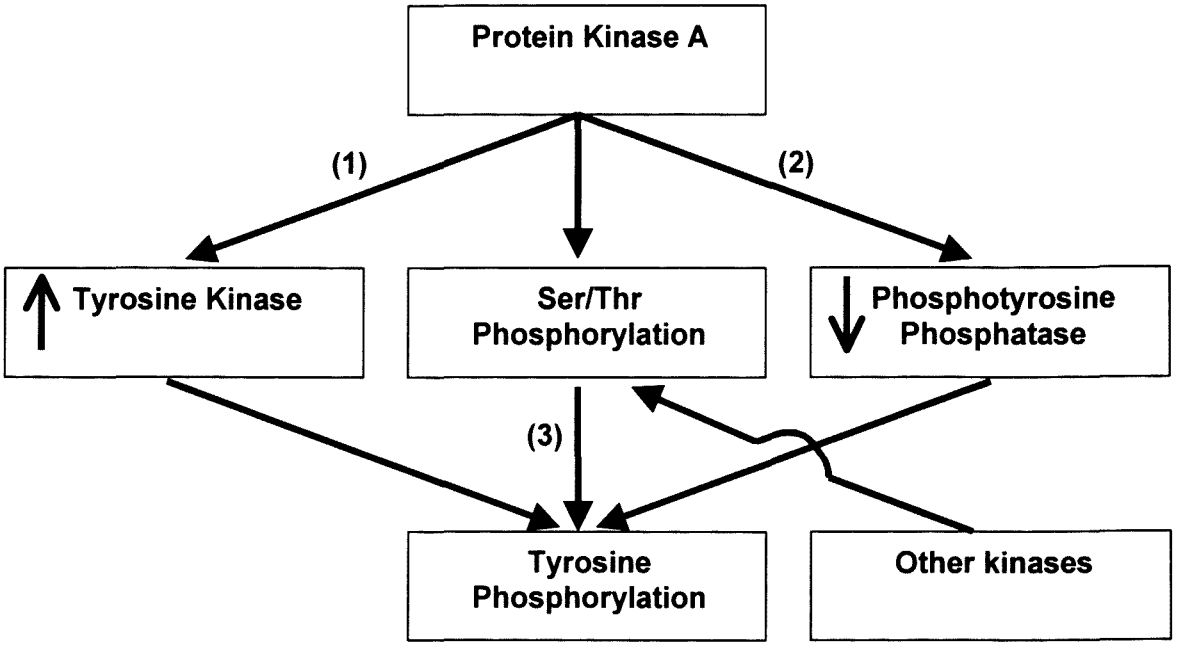

Figure. 3. *Regulation of protein tyrosine phosphorylation by a cAMP/PKA-dependent pathway. (1) A protein tyrosine kinase is stimulated through direct phosphorylation by PKA or by an enzymatic cascade that involves phosphorylation by PKA. (2) A phosphotyrosine phosphatase is inhibited through direct phosphorylation by PKA or by an enzymatic cascade that involves phosphorylation by PKA. (3) Protein targets become substrates for protein tyrosine activity after phosphorylation in ser or thr residues by PKA (Visconti et al., 2002).*

2.2.3.4 Crosstalk between different signalling events during sperm capacitation

Cyclic AMP appears to be a central regulator of several sperm processes, such as motility (Eddy & O'Brien, 1994), capacitation (Visconti *et al.*, 1995b; Visconti *et al.*, 1997), and the AR (De Jonge *et al.*, 1991a; Leclerc & Kopf, 1995; Garde & Roldan, 2000). Changes in membrane potential are also involved in these sperm functions (Morisawa & Suzuki, 1980; Florman *et al.*, 1992; Zeng *et al.*, 1995; Arnoult *et al.*, 1996; Darszon *et al.*, 1999). Presently, it is not known whether these two (capacitation and AR) signalling events interact. However, it is likely that these two processes are related, since capacitation is accompanied by both hyperpolarization of the plasma membrane and an increase in cAMP synthesis. Supporting this idea, the presence of a membrane potential-regulated AC has been reported in non-mammalian species (Beltran *et al.*, 1996). In addition, although PKA is the main downstream effector for cAMP in sperm, the view that PKA mediates all of the effects of cAMP has been amended with the discovery of new types of cyclic nucleotide receptors. These receptors include cyclic-nucleotide-gated channels, exchange factors (Kawasaki *et al.*, 1998), a cGMP binding cyclic nucleotide PDE, and extracellular cAMP receptors (Shabb & Corbin, 1992). Cyclic nucleotide-gated channels were identified in sea urchin (Gauss *et al.*, 1998) and mammalian sperm (Weyand *et al.*, 1994) with specificity for cAMP and cGMP, respectively. Altogether, these data support the idea that crosstalk might occur between modification in membrane potential and the cAMP signalling pathway during capacitation. Alternative possibilities are summarised in Figure 4.

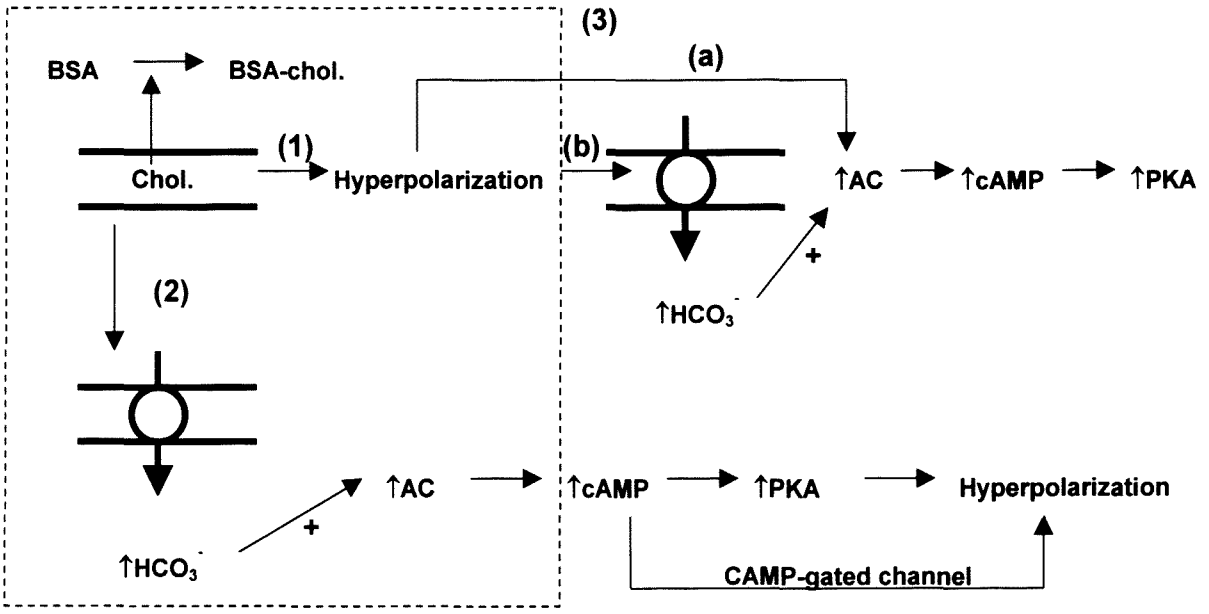

Figure 4. *Crosstalk between signalling pathways involved in capacitation. (1) Hyperpolarization is upstream to the increase in cAMP synthesis. Cholesterol removal regulates sperm plasma membrane potential through a K^+ channel or through the increase in anionic permeability, (a) hyperpolarization may regulate AC activity as described in sea urchin and trout sperm (Beltran et al., 1996; Morisawa & Ishida, 1987) or (b) hyperpolarization may regulate HCO_3^- permeability and in this way activate AC. (2) Hyperpolarization is downstream of the increase in cAMP synthesis. In this model, cholesterol removal regulates an $HHCO_3^-$ permeability and HCO_3^- stimulates AC, cAMP could then activate a cyclic nucleotide gated channel directly or indirectly through phosphorylation by PKA leading to plasma membrane hyperpolarization. (3) Hyperpolarization and cAMP synthesis are independently associated with capacitation.*

2.2.3.5 Consequences of capacitation on sperm function

Although fertilization still represents the endpoint of sperm capacitation, the ability of the sperm to undergo a regulated AR (e.g. in response to the ZP or P) can be taken as an earlier, upstream endpoint of capacitation (Florman & Babcock, 1991; Visconti *et al.*, 1998). Capacitation is also correlated with changes in sperm motility patterns, designated as hyperactivation, in a number of species (Suarez, 1996; Yanagimachi, 1994). When one attempts to understand the process of capacitation at the molecular level, events occurring both in the sperm head (i.e. AR) and in the tail (i.e. motility changes) must be considered. Therefore, one may postulate that components of both the sperm exocytotic and motility machinery are modified during capacitation. Some of these alterations may involve changes in the phosphorylation status of certain proteins, changes in protein localisation, and/or modification of protein-protein interactions. Experiments leading to the identification and characterisation of these effector molecules will further increase our understanding of capacitation.

To understand the link between capacitation and the AR, a better knowledge of the mechanisms that regulate this exocytotic event in sperm is necessary. Exocytosis is a tightly regulated, complex process that involves fusion of subcellular vesicles with the overlying plasma membrane and release of vesicular contents. Recent evidence suggests that membrane fusion is governed by a few conserved protein families regardless of whether membrane fusion occurs between intracellular organelles or between trafficking vesicles and the plasma membrane. Proteins involved in fusion events include a family of proteins commonly referred to as SNARE proteins (soluble N-ethylmaleimide-sensitive attachment protein receptors) (Jahn & Sudhof, 1999).

Sperm homologues of SNARE proteins as well as SNARE-associated proteins, such as Rab 3A and NSF, have been detected in sea urchin (Schulz *et al.*, 1997; Schulz *et al.*, 1998) and mammalian sperm (Michaut *et al.*, 2000; Ramalho-Santos *et al.*, 2000; Yunes *et al.*, 2000). These observations support the idea that the sperm AR might be regulated in similar ways to exocytotic processes in somatic cells. Since capacitation is necessary for exocytosis in mammalian sperm, elucidation of the mechanisms regulating the AR will also increase our understanding of capacitation.

Capacitation is also linked to events that occur in the sperm flagellum. For example, two members of the AKAP family located in the fibrous sheath become phosphorylated at tyrosine residues during human sperm capacitation (Carrera *et al.*, 1996; Mandal *et al.*, 1999; Vijayaraghavan *et al.*, 1999). AKAPs represent a growing family of scaffolding proteins that function to tether the regulatory subunits of PKA and signalling enzymes, such as calcineurin and PKC, to organelles or cytoskeletal elements. These proteins permit the precise control of signal transduction in discrete regions of the cell (Pawson & Scott, 1997). Tyrosine phosphorylation of AKAPs might alter the biochemical and biophysical properties of these proteins and the fibrous sheath and thus, contribute to the regulation of events associated with flagellar bending, including changes in tail wave amplitude during hyperactivation.

2.3 The sperm acrosome

The acrosome plays an important role at the site of sperm-*zona* (egg) binding during the fertilizing process. Clinical studies have identified a distinct group of men whose infertility is associated with abnormal AR (Benoff, 1997). Since the acrosome acts in concert with the plasma membrane overlying the acrosome during the early events of fertilization, any discussion on its formation and organisation will contribute to our understanding of its functional significance.

The acrosome is a Golgi-derived secretory granule that is formed during an early stage of spermiogenesis. It resembles the cellular lysosome, a bag like structure that normally functions in intracellular digestive and defensive mechanisms, in three different ways. First, both the acrosome and the lysosome are derived from the Golgi apparatus. Secondly, both organelles stain a bright orange red colour with acridine orange, indicating an acidic pH within the organelles. Finally the two organelles contain several common enzymes such as acid glycohydrolases, proteases, esterases, acid phosphatases and aryl sulfatases (Tulsiani *et al.*, 1998; Zaneveld & De Jonge, 1991). Despite these similarities the acrosome has some distinctive features. The sperm acrosome is a sac-like structure surrounded by inner and outer acrosomal membranes. Immediately after sperm (receptor)-*zona* ligand) binding, the outer acrosomal membrane fuse with the overlying plasma membrane, releasing the acrosomal contents (glycohydrolases, proteases, etc.) at the site of sperm-egg binding (acrosomal exocytosis). The acrosome is also different from the cellular lysosome in that it contains antigens such as acrosin (Saling, 1989), acrogranin (Anakwe & Gerton, 1990), and sperm protein AM67 (Foster *et al.*, 1997). Because of

these differences and its exocytotic properties, the sperm acrosome is considered analogous to a secretory granule (Eddy & O'Brien, 1994).

Several biochemical and ultrastructural studies have provided evidence for the involvement of cytoskeletal domains such as actin (Talbot & Kleve, 1978), calmodulin (Camatini & Casale, 1987), and α-spectrin-like antigens (Virtanen *et al.*, 1984) in the organisation of the acrosome. In addition, the organelle contains filamentous structures primarily associated with the outer acrosomal membrane (Olson *et al.*, 1987). However the functional significance of the filamentous structures, if any, is not yet known (Abou-Haila & Tulsiani, 2000).

Today, most researchers agree that the powerful hydrolytic enzymes (glycohydrolases, proteases, etc) released at the site of sperm-egg binding, along with the enhanced thrust generated by the hyperactivated beat pattern of the bound spermatozoa (Katz & Drobnis, 1990), are the important factors regulating the penetration of ZP and fusion of the gametes.

2.3.1 The acrosome reaction (AR)

In all mammals, sperm cells are required to fertilize oocytes, thereby providing a haploid set of chromosomes with a paternal pattern of genomic imprinting needed for normal development and triggering oocyte activation (Loeb, 1915; Austin, 1954; Yanagimachi, 1994). It has long been known that successful fertilization is dependent on the extracellular ionic environment, in large part because this can modify the intracellular composition of gametes. The first observation was made in the sea

urchin when it was noted that fertilization did not occur in the absence of extracellular Ca^{2+} (Loeb, 1952) due to failure of the AR to occur. After the development of successful culture systems for mammalian gametes, it was possible to demonstrate that mammalian sperm fertilizing ability, like that of invertebrate sperm, can be modulated by alterations in extracellular components (Fraser, 1995).

The AR is an exocytotic process physiologically induced by ligand (ZP3)-receptor interaction, consisting in multiple fusions between the outer acrosomal membrane and the overlaying plasma membrane leading to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface that mediate fusion with the oolemma. The sperm acrosome is a Golgi-derived structure forming a cap over the anterior region of the nucleus that contains many hydrolytic enzymes and consists of an anterior cap and a posterior region called the equatorial segment (Yanagimachi, 1994). As mentioned previously, only capacitated sperm are physiologically able to undergo the AR in response to physiological stimuli. It is thus conceivable that the two process, capacitation and AR, are sequentially and functionally linked such that several of the effectors involved in mediating intracellular signalling activated by AR start to be tuned during capacitation. For instance, the increase of intracellular calcium levels and tyrosine phosphorylation of proteins accompanying capacitation is also essential for the subsequent exocytosis in response to P (Baldi *et al.*, 1991; Aitken *et al.*, 1998). The AR consists of the development of multiple fenestrations between the outer acrosomal membrane and the plasma membrane of the spermatozoa (Yanagimachi, 1994). This lead to the release of the enzymatic content of the acrosome and to the exposure of the enzymes bound to the inner membrane adjacent to the nuclear envelope

(Yanagimachi, 1994). In the absence of any specific stimuli only a low percentage of human spermatozoa can undergo the AR (Leyton & Saling, 1989). It has been suggested that self-aggregation of the sperm receptor for ZP may account for this spontaneous acrosome reaction (Saling, 1989). A wide variety of molecules present on the surface of the sperm have been proposed as putative candidates for the ZP3 receptor (Wassarman, 1999). The involvement of different receptors in different species or different binding affinities, or even multiple receptors that may co-operate in sequence to induce AR may justify this rather long list of possible candidates. However, none of these molecules has been definitively recognised as "the sperm-egg receptor" (Wassarman, 1999). Progesterone and a highly conserved ZP glycoprotein termed ZP3 have been identified as natural oocyte-associated AR-inducing ligands, and their sequential action has been shown to support the occurrence of the physiological AR (Melendrez *et al.*, 1994; Roldan *et al.*, 1994). There are also apparent divergences between the two pathways because the one used by the ZP ligand involves a pertussis toxin sensitive G-protein (Franken *et al.*, 1993) whereas that used by P does not (Tesarik *et al.*, 1993). Moreover, P also stimulates transmembrane chloride fluxes employing a plasma membrane channel sharing some, but not all properties with neuronal γ-aminobutyric acid A ($GABA_A$) receptor (Wistrom & Meizel, 1993; Blackmore *et al.*, 1994; Shi & Roldan, 1995).

It is well known that P opens a sperm plasma membrane calcium channel and activates phosphotyrosine kinase independently of each other (Mendoza *et al.*, 1995). Progesterone therefore reacts with a multiple-receptor system on the surface and this system co-operates with that used by ZP3 to control the physiological AR.

Each of the respective receptors alone can eventually induce some of the AR events and in some cases complete acrosomal exocytosis.

The AR can also be physiologically induced by P. It is present at high levels in the cumulus matrix, surrounding the oocyte, that must be crossed by sperm in order to reach the ZP. This steroid has been described to affect several other sperm functions, including capacitation, motility and the priming effect on the ZP3-induced AR through stimulation of a rapid nongenomic signalling pathway mediated by P receptors present on the sperm surface (for further details on effects of P see Baldi *et al.*, 1998; Bray *et al.*, 1999). P-induced AR is inhibited by 17b-estradiol through interaction with a specific nongenomic estrogen receptor on the sperm plasma membrane (Luconi *et al.*, 1999). This suggest that estradiol, which is present at micromolar levels in the follicular fluid, may act as a physiological modulator of P action on sperm assuring the appropriate timing of activation in the fertilization process. Other agonists proposed to act as *in vivo* inducers of the AR are listed in Table 1.

Table 1. *Molecules that can induce the acrosome reaction in vitro.*

AGONIST	**REFERENCE**
Zona pellucida protein 3 (ZP3)	Yanagimachi, 1994; Saling, 1989
Prostaglandin E1	Schaefer *et al.*, 1998
ATP	Foresta *et al.*, 1993
Dibutyryl cAMP (cAMP analogues)	De Jonge *et al.*, 1991b
Progesterone & 17bOH-progesterone	Baldi *et al.*, 1998; Bray *et al.*, 1999
Platelet-activating factor	Sengoku *et al.*, 1992; Krausz *et al.*, 1994;
Atrial natriuretic peptide	Breitbart & Naor, 1999
Epidermal growth factor	Furuya *et al.*, 1993
Serum albumin	Yanagimachi, 1994
Manosylated bovine serum albumin	Benoff *et al.*, 1997a
A23187	Fènichel *et al.*, 1989
GABA	Shi *et al.*, 1997
Forskolin	De Jonge *et al.*, 1991b
Pentoxifylline	Gearon *et al.*, 1994
1-oleoyl-2-acetylglycerol (DAG analogue)	Rotem *et al.*, 1992
Phorbol diesters (PMA, TPA)	De Jonge *et al.*, 1991b; Lee *et al.*, 1987
Thapsigargin	Meizel & Turner, 1993

Many molecular mechanisms have been demonstrated to be active during AR (Figure 5), but for many of them a precise cause-effect relationship has not yet been defined, such that we still do not know whether they are essential for the process of fertilization or whether they are simply associated with the process. The fundamental difference between P- and ZP3-induced AR stands on the nature of their receptors. In fact, in the case of ZP3, its action is mediated by receptor recruitment of G-proteins which are not involved in a P stimulated cascade (Wassarman, 1999). One of the first events that occur in spermatozoa following stimulation with ZP3 and P is receptor aggregation (Saling, 1989; Tesarik & Mendoza, 1993). This is followed by a cascade of downstream membrane and cytosolic signalling factors involved in induction of AR (schematised Figure 5). Among them, the roles of calcium, phospholipases and protein kinases are discussed below.

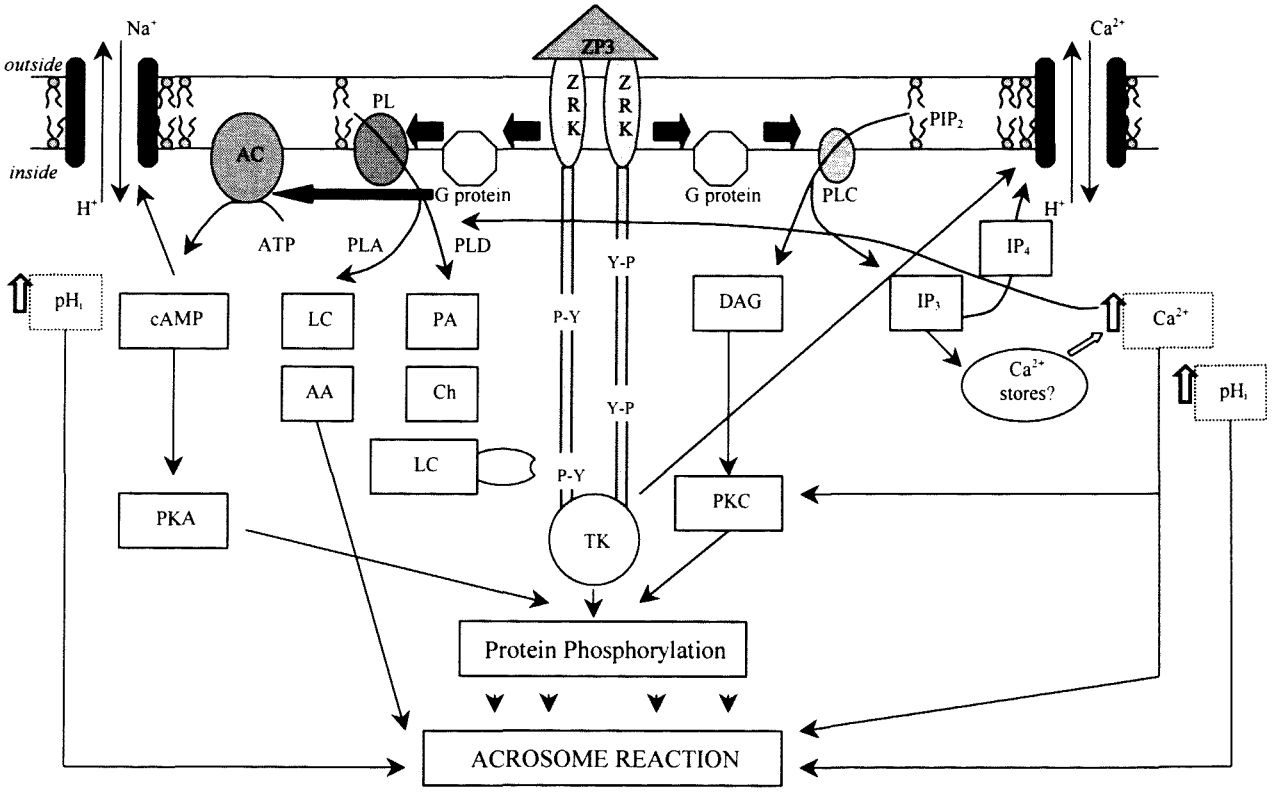

Figure 5. *Diagram illustrating the main signal transduction pathways activated during the process of AR in response to zona protein 3 (ZP3). Following interaction with the agonist, aggregation of receptors for ZP3 (ZRK) induces TK activation (which increases protein tyrosine phosphorylation) and autophosphorylation of the receptor. Activation of phosphatidylinositol-3kinase (PI3K) has been also reported. A guanine nucleotide binding protein (G-protein) transduces the signal interacting with membrane-bound enzymes like phospholipase C (PLC) and AC. Activation of these two enzymes lead to increased generation of the second messengers cyclic adenosine monophosphate (cAMP), IP_3 and diacylglycerol (DAG). A consequence of the increase of second messengers is the activation of protein kinases such as cAMP-dependent kinase (PKA) and Ca^{2+} and phospholipid-dependent kinase (PKC) with increased protein phosphorylation. cAMP-dependent influx of sodium (Na^+) has*

been reported. IP_3 may increase intracellular Ca^{2+} by liberation of the ion from intracellular Ca^{2+} stores. The increase of intracellular Ca^{2+} consequent to activation of ZP3 receptors is completely due to influx from the extracellular medium, is dependent on activation of G-proteins, involves voltage-dependent Ca^{2+} channels and is accompanied by an efflux of H^+ which determines a rise of intracellular pH (pHi). Partial dependence of Ca^{2+} -influx from TK activation has been reported. Ca^{2+} dependent activation of phospholipase A2 (PLA2) and phospholipase D (PLD) [with increased generation of other second messengers as arachidonic acid (AA), lyso-phosphatidylcholine (LC) and phosphatidic acid (PA) from membrane phospholipids (PL)] have also been described to occur during AR. (From: Baldi et al., 2000)

2.3.1.1 Increase in intracellular calcium during acrosome reaction

Calcium plays a central role in receptor-mediated response and membrane fusion processes in spermatozoa (Yanagimachi, 1994). Calcium ionophores are the most widely used non-physiological inducers of AR (Yanagimachi, 1994). Although the AR can be induced in the absence of extracellular calcium with some agonists (Foresta *et al.*, 1993; Krausz *et al.*, 1994; Bielfield *et al.*, 1994) stimulation of calcium fluxes is one of the earlier responses activated by most stimuli that induce the AR. The shape of the calcium wave is different during P- and ZP- stimulation. P elicits a biphasic calcium wave, consisting of a rapid initial peak followed by a long lasting plateau phase (Baldi *et al.*, 1998). While ZP3 induce a slow and sustained increase of calcium (Florman, 1994), resembling the second phase of P. However, it has been shown, using a different methodological approach, that ZP3 may induce a rapid increase of intracellular calcium concentrations occurring within milliseconds after stimulation (Arnoult *et al.*, 1999). Parallel inhibition of both AR and the second phase

of calcium response to P by both TK blockers and a previous administration of estradiol, suggests the plateau phase to be responsible for agonist- induced AR (Bonaccorsi *et al.*, 1995; Tesarik *et al.*, 1996; Bray *et al.*, 1999). Recently, a direct relationship between the sustained calcium phase and AR in response to ZP3 has been demonstrated (O'Toole *et al.*, 2000). Concerning P, such a relationship is less apparent, since the percentage of sperm undergoing AR in response to the steroid is smaller than that where a sustained response is observed (Kirkman-Brown *et al.*, 2000; Kobori *et al.*, 2000). Although extracellular calcium depletion totally prevents both ZP3 and P-induced intracellular calcium increase, recent data suggest the possible involvement of intracellular calcium stores in sperm the AR (Walensky & Snyder, 1995; Dragileva *et al.*, 1999; O'Toole *et al.*, 2000). In particular, it has been shown that calcium entry during the sustained phase in response to ZP3 is due to activation of store-operated channels (O'Toole *et al.*, 2000). While membrane voltage-operated calcium channels of T-type have been demonstrated in mediating ZP-induced calcium influx (Arnoult *et al.*, 1996; Darszon *et al.*, 1999), the nature of P-stimulated calcium channels is still a matter of discussion (Garcia & Meizel, 1999; Blackmore & Eisoldt, 1999; Patrat *et al.*, 2000). Calcium plays a key role in the fusion events in the sperm membrane (Watson *et al.*, 1995). Using a pyroantimonate-osmium fixation technique, the temporal and spatial location of intracellular calcium granules was monitored during AR in ram spermatozoa (Watson *et al.*, 1995). Ca^{2+} is initially associated with the outer acrosomal membrane. As the process progresses, Ca^{2+} associates with the fusion sites between the outer acrosomal membrane and the plasma membrane anteriorly to the equatorial segment. At later stages, Ca^{2+} is localised in both post acrosomal dense lamina and on the outer acrosomal

membrane under the equatorial segment. These findings suggest that Ca^{2+} may be implicated in the fusion process (Watson *et al.*, 1995).

Increase of intracellular Ca^{2+} in response to ZP3 and P is associated with an efflux of H^+ and a rise in intracellular pH (Florman *et al.*, 1989; Garcia & Meizel, 1996; Brook *et al.*, 1996). P has been shown to rapidly stimulate sodium influx (Patrat *et al.*, 2000) and the presence of sodium in the extracellular medium is absolutely required for induction of the AR by the steroid (Patrat *et al.*, 2000; Garcia & Meizel, 1996). Sperm intracellular pH may be regulated by a Na^+/H^+ exchanger (Garcia & Meizel, 1999) as well as by Cl^-/HCO_3^- (Holappa *et al.*, 1999), whose presence have been demonstrated in germ cells.

2.3.1.2 Phospholipase activation during acrosome reaction

The presence of PLA2, PLC and other phospholipases have been demonstrated in mammalian spermatozoa (Roldan, 1998). The roles and activities of these enzymes in human sperm capacitation and AR have been recently reviewed (Roldan, 1998). It was also shown previously that during the AR in boar spermatozoa, the amount of DAG and free fatty acids increases (Nikolopoulou *et al.*, 1986). This finding was consistent with activation of phospholipases (Roldan, 1998). In fact, increase of $[Ca^{2+}]i$ stimulated by ionophores and P leads to activation of PLC in human spermatozoa (Roldan, 1998). Such activation leads to an intracellular increase in the amount of IP_3 and DAG. Similarly, ZP3 activates the phosphoinositide specific enzyme phospholipase Cg1 by virtue of tyrosine phosphorylation and leads to its translocation from cytosol to particulate fractions (Tomes *et al.*, 1996). Presence and activation of phosphatidylcholine-specific PLC during the AR has also been

demonstrated (Roldan, 1998). DAG and IP_3, produced by calcium-dependent activation of PLC, may be respectively involved in the regulation of PKC activity and release of calcium from putative sperm intracellular stores (Walensky & Snyder, 1995; Breitbart & Noar, 1999).

AR induced by ionophores and P also leads to the activation of PLA2 (Roldan, 1998). This activation is associated with generation of lipid metabolites, such as arachidonic acid and lysophospholipids. Phosphatidylcholine, lysophospholipids, and unsaturated fatty acids, such as arachidonic acid, are potent inducers of AR (Cross, 1994; Fleming & Yanagimachi, 1981; Kyono *et al.*, 1984) and may be implicated in the fusion process that occurs during AR. Moreover, LC generated from PLA2 activation may act as a substrate for generation of platelet-activating factor (PAF) (Baldi *et al.*, 1993; Kumar *et al.*, 1988). This phospholipid, which is synthesised in response to P (Baldi *et al.*, 1993), may further enhance AR (Krausz *et al.*, 1994; Fukuda *et al.*, 1994; Huo & Yang, 2000). *In vitro* treatment of human sperm with PAF (1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine) enhances penetration of *zona* free hamster oocytes (Minhas, 1993). In animal studies, *in vitro* treatment of sperm with PAF significantly improves fertilization rate using both intra cytoplasmic sperm injection (ICSI) and in vitro fertilization (IVF) techniques without showing detrimental effects on subsequent embryo development (Minhas *et al.*, 1996; Lee *et al.*, 1997).

2.3.1.3 Involvement of protein kinases in acrosome reaction process

During AR, activation of protein kinases (serine-threonine kinases as PKC, PKA and protein kinase G [PKG], and TKs) is downstream to the production and/or activation of early second messengers. Early evidence for the involvement of PKA in the AR,

demonstrated that AC activity and cAMP generation increase during this process (De Jonge *et al.*, 1991a). Adenyl cyclase stimulators, such as forskolin, and the cAMP analogue dibutyryl cAMP, induce AR in a dose-dependent manner in mammalian sperm (Anderson *et al.*, 1992; Garde & Roldan, 2000; Harrison & Meizel, 2000; Kobori *et al.*, 2000). In addition, PKA inhibitors have been reported to suppress the AR induced by P (Harrison & Meizel, 2000), although controversial results are present in the literature concerning an increase of cAMP synthesis in response to P (Parinaud & Milhet, 1996; Schaefer *et al.*, 1998). Taken together, these findings suggest that PKA activation may be involved in the AR process (Baldi *et al.*, 2000).

The involvement of PKC in the process of AR is still under debate. Although activators such as phorbol esters and synthetic DAG induce the AR, PKC activity in human sperm is very low and the identity of PKC substrates is still under investigation. Induction of AR by solubilized ZP was partially reduced by pre-treatment with inhibitors of PKA, PKC and PKG tested separately, while combinations of them caused a significantly greater inhibition (Bielfield *et al.*, 1994). These results suggest a concomitant role for PKA, PKC and PKG in the human ZP-induced AR (Bielfield *et al.*, 1994). Both P (O'Toole *et al.*, 1996; Bonaccorsi *et al.*, 1998) and ZP (Liu & Baker, 1997) increase sperm PKC activity. Such activation is downstream to calcium influx (P (Bonaccorsi *et al.*, 1998), since it can be prevented by calcium channel blockers (O'Toole *et al.*, 1996).

ZP3 induced an increase in tyrosine phosphorylation of sperm proteins (Leyton & Saling, 1989; Burks *et al.*, 1995). A major tyrosine phosphorylated protein in mammalian spermatozoa was 95-97 kDa (Leyton & Saling, 1989; Burks *et al.*, 1995).

Proteins in this molecular weight range appear to undergo phosphorylation during capacitation (see above) and in response to P (Baldi *et al.*, 1998). Recombinant human ZP3 produced by genetic engineering has been demonstrated to induce AR and tyrosine phosphorylation of the 95-110 kDa protein in human sperm (Brewis *et al.*, 1998), perfectly mimicking the physiological action of this glycoprotein. Known inhibitors of TK, such as genistein and tyrphostin-47, block ZP3-induced AR (Leyton *et al.*, 1992). Both the TK inhibitors tyrphostin A48 and pertussis toxin suppress ZP3-induced calcium influx in mouse spermatozoa (Bailey & Storey, 1994). Using a similar pharmacological approach, Tesarik *et al.* (1993) and Luconi *et al.* (1995) showed involvement of TKs in the P-mediated AR. Activation of TK also seem to be involved in the plateau phase of increase in the amount of intracellular Ca^{2+} in response to P (Bonaccorsi *et al.*, 1995; Tesarik *et al.*, 1996). The mechanisms through which TKs are activated in sperm during the process of AR are still under investigation. Although during capacitation activation of the AC/cAMP/PKA pathway appears to be highly involved, activation of this pathway in response to stimuli that induce AR is less clear (Parinaud & Milhet, 1996; Schaefer *et al.*, 1998). The involvement of reactive oxygen species, generated by sperm in response to stimuli that induce AR, in tyrosine phosphorylation has been suggested by a few researchers (de Lamirande *et al.*, 1998; Fisher *et al.*, 1998).

Recent data (Fisher *et al.*, 1998) indicate the involvement of PI3K in the AR stimulated by agents that mimic ZP3. These include mannose-BSA and polyclonal antibodies raised against the p95 protein (indicated as the possible sperm ZP3 receptor; see ZRK above), but not by P or ionophores (Baldi *et al.*, 2000).

2.4 Motility

Human spermatozoa emerge highly differentiated from the testes, but as for all other eutheria, they are motionless or feebly motile. This immotility is apparently due to the "immaturity" of the plasmalemma, as demembrated spermatozoa can be induced, under appropriate conditions to move almost as actively as mature spermatozoa from the cauda (Mohri & Yanagimachi, 1980). The spermatozoa continue to develop during their passage through the epididymis. Only at ejaculation, when they are mixed with the secretions of the accessory glands, do spermatozoa undergo motility activation, acquiring a mature motility pattern and fertilizing ability.

Sperm motility plays an important role in transport of the spermatozoa in the female reproductive tract before fertilization (Yanagimachi, 1969). Sperm motility is a complex phenomenon, the understanding of which requires integration of cell biology with reproductive physiology, biochemistry, biophysics and clinical andrology. The spermatozoon is an intricate motile cell, whose motility depends on a flagellum that develops the propulsive force for swimming. The likelihood of achieving a pregnancy increases with decreasing proportions of immotile spermatozoa and with increasing quality of sperm progression (Bostofte *et al.*, 1983; Bostofte *et al.*, 1984).

Over the years a plethora of terminology has appeared to describe the various movement characteristics of motile spermatozoa. Consensus on these parameters was reached at the "Automated Sperm Motility Analysis" workshop held at the American Society of Andrology's Annual Meeting in Houston (Tx, USA) during March 1988 (Mortimer 1990). These parameters have now been standardised and are currently used in motion analysis (Owen & Katz, 1993). These parameters include:

Curvilinear velocity (VCL): The VCL (µm/s) is the time-average velocity of a sperm head along its actual curvilinear path.

Straight line velocity (VSL): The VSL (µm/s) is the time-average linear velocity of a sperm head along the straight line between the start and the end of the observed track.

Average path velocity (VAP): The VAP (µm/s) is the time-average velocity of a sperm head along its average path. This path is computed by smoothing the actual path according to algorithms in the CASA instrument.

Amplitude of lateral head displacement (ALH): The ALH (µm) is the magnitude of lateral displacement/deviation of a sperm head about its average path. It can be expressed as a maximum or an average of such displacements.

Beat-cross frequency (BCF): The BCF (Hz) is the average rate at which the sperm's curvilinear path crosses its average path.

Straightness (STR): The STR (%) is the ratio of VSL/VAP and measures the straightness of the average path.

Linearity (Lin): The Lin (%) is reported as the ratio of VSL/VCL and measures the linearity of the curvilinear path. The value range from 0 to 100 with a value of 100 representing cells swimming in a straight line.

All these motility parameters, as depicted in Figure 6, give a clear indication of movement characteristics of spermatozoa (WHO, 1999).

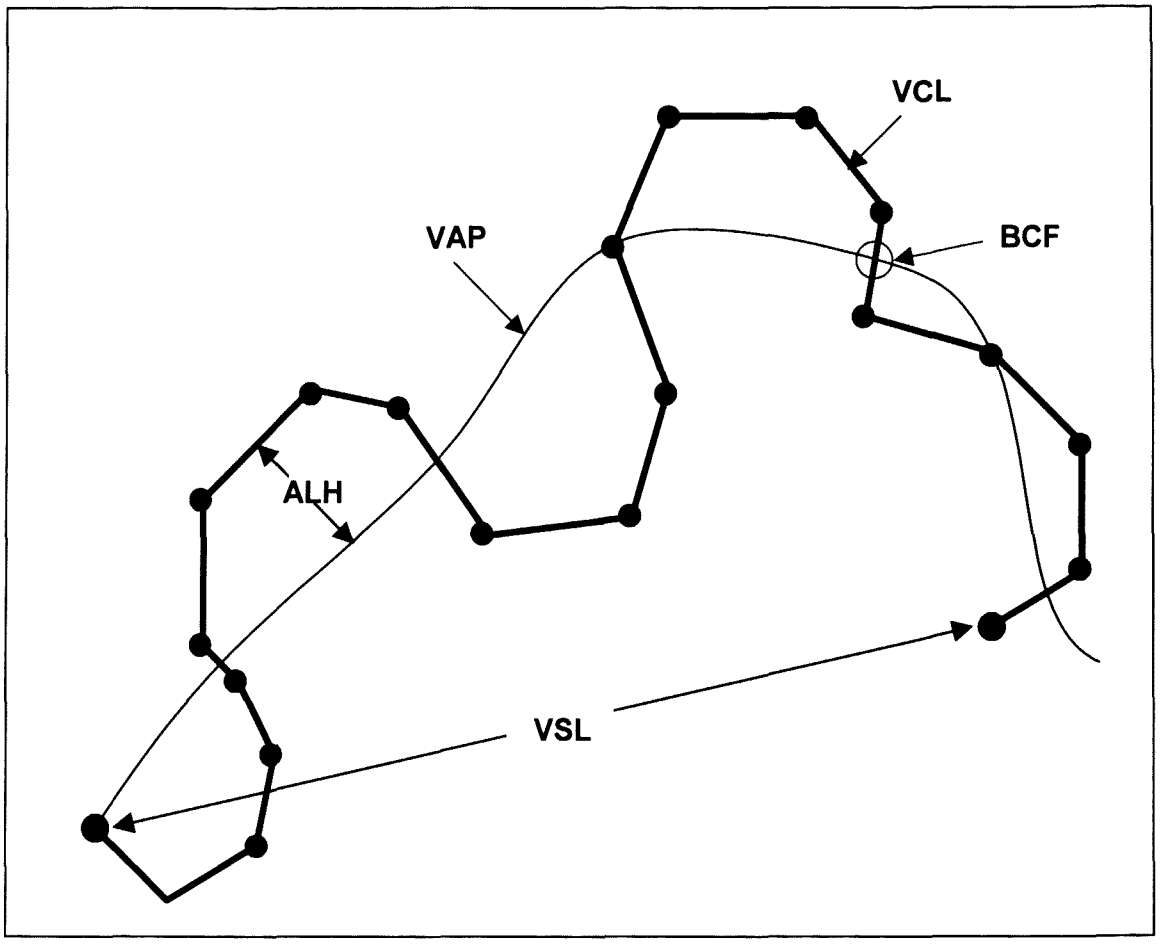

***Figure 6**: Motion parameters of a single sperm track, where ALH = amplitude of lateral head displacement; BCF = beat-cross frequency; VAP = average path velocity; VCL = curvilinear velocity and VSL = straight line velocity.*

2.4.1 Factors influencing sperm motility

Enhanced sperm motility values have been associated with improved conception rates (Edvinsson *et al.*, 1983). In this regard, efforts have been directed towards improvement of poor motility of sperm as well as the inducement of motility of immotile cells. The practice of mixing sperm with a motility stimulating agent for improving the successful rate of artificial insemination has been reported (Yovich *et al.*, 1988).

2.4.1.1 Cyclic adenosine mono-phosphate (cAMP)

Cyclic nucleotides play a major role in sperm capacitation, metabolism, AR and motility (Tash & Means, 1983). This was first observed when it was noted that caffeine and other methyl-xanthines, which act as cyclic nucleotide PDE inhibitors and associated with an increase in intracellular cAMP, can stimulate sperm motility and metabolic activity (Hoskins, 1973).

Garbers *et al.* (1971) first showed that the addition of dibutryl cAMP stimulated the motility of bovine caudal epididymal spermatozoa. cAMP was shown to have effects on the metabolism and motility of sperm (Tash & Means, 1982). Since then the cAMP system of spermatozoa has been extensively studied.

Elevation of intracellular cAMP levels appear to be related to:

...the acquisition of the potential for motility during epididymal maturation;

...the actual initiation of motility upon ejaculation; and

...the surge of increased motility associated with capacitation.

The cAMP content of diluted spermatozoa increases two fold as sperm traverse the epididymis (Hoskins *et al.*, 1974). This seems reasonable since the intracellular concentrations of cAMP correlates directly with the degree of motility (Hoskins & Casillas, 1975). Furthermore, a 25% decrease in cAMP levels is observed as washed bovine caudal sperm become immotile as compared to the initial motility (Hoskins *et al.*, 1974). During ejaculation, as bovine caudal epididymal spermatozoa are mixed with secretions from the accessory glands, cAMP levels double within 30 seconds (Cascieri *et al.*, 1976). A similar rise in cAMP concentration has been reported in hamster spermatozoa (Morton *et al.*, 1974).

cAMP has also been implicated with sperm motility during capacitation and AR (Rosado *et al.*, 1974). Vigorous motility is commonly observed concomitant with capacitation and is believed to be essential for penetration of the egg following the AR (Yanagimachi, 1970). Reyes *et al.* (1977) showed that the addition of exogenous cAMP to rabbit or human spermatozoa more than doubled the rate of capacitation *in vitro*.

cAMP appears to activate cAMP dependant kinase, which phosphorylates key proteins that are required for motility (Lindemann & Kanous, 1989). Deactivation occurs when these proteins are dephosphorylated by phosphoprotein phosphatase. The phosphorylation of several integral axonemal proteins has been reported to be cAMP-dependant (Tash *et al.*, 1984; Horowitz e*t al.* 1988).

2.4.1.2 Adenylate cyclase

Mammalian sperm possess both AC and PDEs, which are involved in the regulation of the intracellular concentration of cAMP (Bhatnagar *et al.,* 1982) and also possess cAMP-dependant protein kinase through which the physiological action of cAMP is mainly mediated. Pariset *et al.* (1983) reported that there is a positive correlation between the motility index and the cAMP-dependant protein kinase activity of human spermatozoa.

A significant positive correlation was found between sperm motility and cAMP content and AC activity in human spermatozoa (Ishikawa *et al.*, 1989). These results suggest that sperm motility is controlled by the AC activity in spermatozoa and that the disturbance of sperm motility in infertile men is probably caused by reduced activity of AC.

2.5 Summary

Mammalian sperm are not immediately fertile upon release from the male reproductive tract, despite their ability to exhibit vigorous motility. They require a species dependent period of time during which they undergo a series of changes, collectively referred to as capacitation (Austin, 1952; Yanagimachi, 1994), that are needed for cells to become fully competent to fertilize an oocyte. When capacitated, mammalian sperm can (i) express hyperactivated motility, the very vigorous, thrusting pattern of motility that is needed for penetration of the oocyte investments and (ii) interact with the oocytes (including cumulus cells, follicular fluid and ZP) to undergo the AR. Capacitation is a unique feature of mammalian sperm that occurs in the female tract and is essential for fertilization. During capacitation, sperm acquire

hyperactive motility and the ability to undergo a regulated AR. Capacitation is accompanied by changes in lipid composition of the plasma membrane as well as by protein post-translational modifications regulated by several signalling pathways.

Studying *in vitro* capacitation has allowed characterisation of a number of biochemical events that occur in human spermatozoa. It is not clear whether the same or similar events occur during capacitation *in vivo*. P, PAF and other effectors present in the follicular fluid and/or cumulus matrix, could facilitate capacitation or prime the AR *in vivo*. For instance, the clinical significance of P effects has been shown by several studies showing a significant correlation of response to P with sperm fertilizing ability as well as reduced or absent response to P in infertile subjects (Baldi *et al.*, 1998; Bray *et al.*, 1999). Moreover, treatment of human sperm with P has been shown to enhance hamster oocyte penetration (Aitken *et al.*, 1996). These studies indicate that sperm P receptor, as well as the one that mediates ZP3 stimulation, is involved in the process of fertilization representing a possible target for developing pharmacological strategies to potentiate sperm fertilizing ability in assisted reproductive techniques or contraceptive molecules. Functional P receptors on the human sperm surface have been recently characterised (Luconi *et al.*, 1998; Falkenstein *et al.*, 1999). Sequencing of the receptor and cloning of the encoding gene will represent next steps in such characterisation.

The AR is an exocytotic event that promotes interaction and penetration through the ZP and confers fusogenic properties on the remaining plasma membrane in the sperm head (Yanagimachi, 1994).

It has been suggested that the importance of capacitation may actually be to prevent sperm from becoming fertile too quickly, given that spermatozoa are deposited into the lower regions of the female reproductive tract and still need to travel some considerable distance in order to reach the site of fertilization (Bedford, 1983). The AR must therefore also be accurately timed to ensure fertilization, since a premature AR leads to the loss of ZP recognition sites from the sperm surface and thus impairs sperm-*zona pellucida* binding (Franken *et al.*, 1993). On the other hand inability of *zona* bound spermatozoa to activate the AR also prevents *zona* penetration.

Although work emanating from multiple laboratories is leading to a better understanding of capacitation, AR and motility, several of the proteins involved in these processes remain to be characterised.

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"All life is an experiment."

- Ralph Waldo Emerson -

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

The material and methods used for each study is outlined in the individual articles as presented in the following chapters. The methods differed slightly between the four studies, however the basic, common and shared materials and procedures used will subsequently be discussed.

3.2.1 Preparation of Human tubal fluid (HTF) culture medium

This simple culture medium was developed from the composition of human oviduct fluid (Quin *et al.*, 1985) and has proved useful for *in vitro* studies and clinical applications including IVF, GIFT and IUI. Chemicals used should be at least of analytical grade and the water to be used of tissue culture grade. The recipe for the preparation of HTF are as follows:

1. Dissolve the following chemicals in about 600ml tissue culture grade water in a 1000-ml volumetric flask: 5.938g NaCl; 0.350g KCl; 0.049g $MgSO_4.7H_2O$; 0.050g KH_2PO_4; 2.100g $NaHCO_3$; 0.036g Na pyruvate; 0.501g Glucose; 0.003g Phenol red; 3.136 ml Na lactate (60% syrup)
2. Separately dissolve 0.300g $CaCl2.2H_2O$ in 100ml tissue culture grade water and add slowly to the rest.
3. Add Penicillin/Streptopen (75mg)
4. Make up to 1000ml with additional culture grade water and mix thoroughly.
5. Adjust pH to 7.5-7.6
6. Check that osmolarity is between 280-290 mOsm.

7. Filter-sterilise into plastic containers under positive pressure.
8. Store at 4°C
9. Warm to 37°C before use

Add 3% BSA if the medium is to be used as a capacitation medium.

3.2.2 Semen collection

Semen samples were obtained from patients attending the *in vitro* fertilization programme at Tygerberg hospital and from healthy donors. All semen samples were collected by masturbation after 2-3 days of sexual abstinence (WHO, 1999). Collections were made in sterile plastic containers after which the semen was allowed to liquify for 30 minutes at 37°C.

3.2.3 Oocyte collection and storage

All oocytes used were nonliving, with no developmental potential. Oocytes were obtained from ovarian tissue that was collected post mortem. This procedure fully complies with the legal and ethical guidelines of the South African Medical Research Council. Ovarian tissue was excised within 24 hours of death and subsequently macerated/manually dissected (Overstreet *et al.*, 1980) and flushed with phosphate buffered saline (PBS, GIBCO, Grand Island, NY). The PBS flushing was stored at 4°C for no longer than 48 hours. *Zona* intact oocytes denuded of granulosa cells were recovered under microscope and placed directly into a 1.5M solution of $MgCl_2$ and stored at 4°C under mineral oil (E.R. Squib & Sons Inc, Princeton, NJ) for immediate use. Oocytes were also stored in a dimethylsulfoxide/sucrose (DMSO) solution at –196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty four hours prior to

each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25M sucrose and 3% BSA in HTF.

3.2.4 Solubilized *zona pellucida* preparation

On the day of the experiment, 20 oocytes were placed in a drop on a petri dish with a glass drawn Pasteur micro pipette, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 20 oocytes on the petri dish. A total volume of 5µl of 10mM HCL was then added to the oocytes on the petri dish in order to dissolve the *zonae* at room temperature. Solubilization of the ZP was microscopically observed and controlled (45-60min). Following solubilization, the reaction was neutralised by adding 5µl of 10mM NaOH rendering a final volume of 10µl. Solubilized ZP solutions with concentrations of 2 ZP/µl were stored up to one week at 4°C.

3.2.5 Assessment of the acrosome reaction

Spermatozoa from the different experiments were fixed on separate spots of spotted slides (MAGV, Germany, XER 201B) and air-dried. Each spot was flooded with fluorescein-labeled *Pisum Sativum* agglutinin solution (FITC-PSA; 125µg/ml; L-0770; Sigma, Cape Town, South Africa) and left in the dark for 30 minutes. After 30 minutes of incubation the excess FITC-PSA was rinsed of in a beaker containing distilled water. Mounting medium and a cover slip was placed on the slides and then evaluated within 24 hours under a fluorescence microscope. Two individuals using the blind scoring method microscopically scored a minimum of 200 spermatozoa for each different point. The following staining patterns were evaluated as acrosome reacted spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in

the equatorial region occurring as an equatorial bar and (iii) no staining over the entire sperm surface. Spermatozoa with patchy FITC-PSA staining were classified as a population of sperm where the acrosome reaction was initiated and all were classified as acrosome reacted (Figure 1).

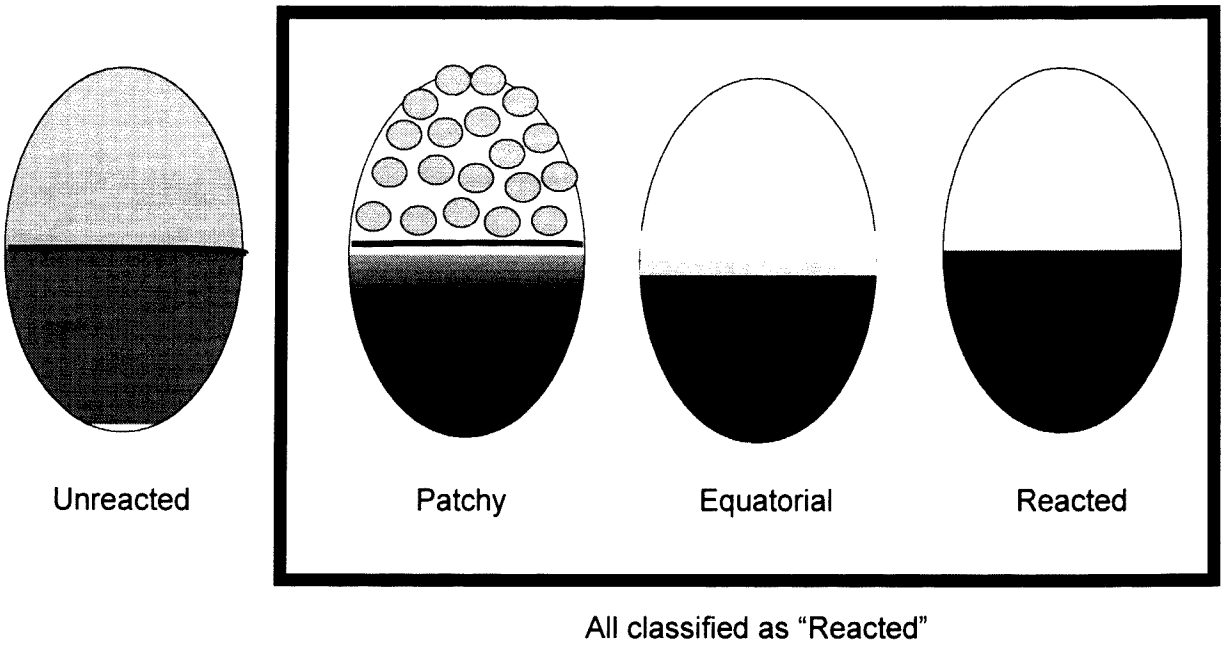

Figure 1: *Patterns recorded during FITC-PSA acrosome staining procedures.*

3.2.6 Hemi*zona* binding assay

This functional assay assesses tight binding of sperm to the outer surface of the *zona pellucida* hemisphere. It allows a controlled comparison of sperm-*zona* binding between control and test spermatozoa, as the *zonae* surfaces are functionally equal.

3.2.6.1 Bisecting of oocytes

A complete micromanipulation system (Narishige, Tokyo, Japan) was used for bisecting the oocytes. An inverted phase-contrast microscope (Nikon Diaphot, Garden City, NY) was equipped with a pair of Narishige micromanipulators (model MO 102), the connecting tubing filled with mineral oil, and a #11 microscalpel blade attached to the manipulator. A 100mm petri dish (Falcon #25382, Falcon plastics, Oxnard, CA) served as the cutting chamber. Culture medium was poured into the dish to a depth of 3mm. Horizontal grooves were cut into the plastic in order to provide support in holding the egg while cutting. The egg was transferred to the working area of the dish using a finely drawn Pasteur glass pipette. Using a total magnification of 200x, the blade was centred and then slowly lowered, first partially flattening the egg and then finally initiating a midline cut into the zona. A further lowering of the blade, along with 1 to 2 side to side excursions, produce two cleanly cut hemi*zonae*. Vigorous pipetting was used to then dislodge the dense ooplasma inside each hemizona. Only one egg was cut at a time to ensure that matched hemi*zona* remained paired for subsequent sperm binding tests. Each hemi*zona* pair was placed in a 50µl droplet of medium in a petri dish, covered with mineral oil and stored at 4°C.

3.2.6.2 Competitive sperm-binding to the hemizona

A 50µl sperm droplet containing $0.5x10^6$ sperm/ml from both the test and control samples were placed in a marked tissue culture dish. One hemi*zona* was placed in the one droplet and the matching hemi*zona* in the other (Figure 2). The droplets were covered with mineral oil in order to prevent evaporation and dehydration of the sperm droplets and co-incubated at 37°C and 5% CO_2 for 4 hours. After co-incubation, the hemi*zona* from each droplet was transferred into culture medium (HTF) droplets respectively. The hemi*zonae* were then rinsed by vigorously pipetting five times with a finely drawn glass pipette in order to dislodge loosely associated spermatozoa. The number of spermatozoa firmly bound to the outer surface of each hemi*zona* was counted under phase contrast microscopy (Nikon TMS-F, Research Inst. Johannesburg, South Africa) at a 200x magnification. The number of sperm bound in the test sample was divided by the number of sperm bound in the control sample and expressed as a percentage to give a hemi*zona* index (HZI).

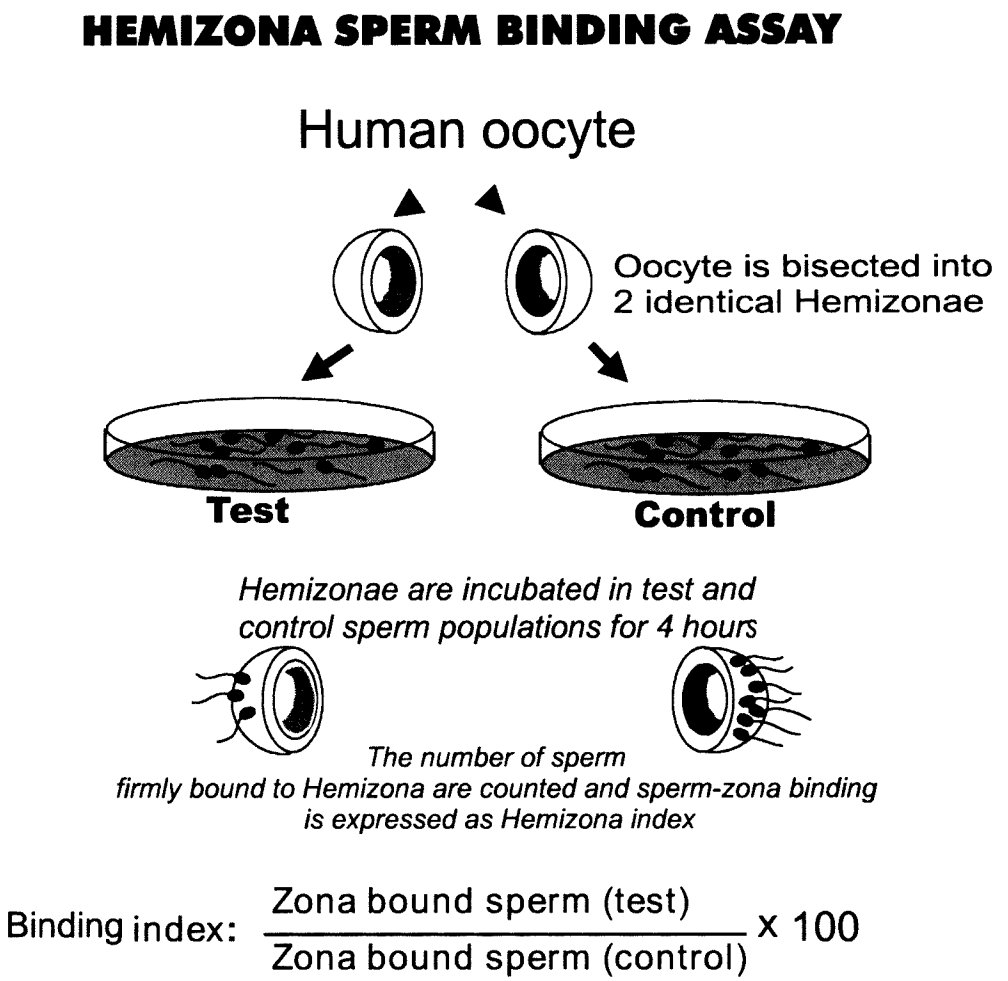

Figure 2: *The competitive hemizona sperm binding assay.*

3.2.7 Computer assisted semen analysis (CASA)

Sperm motility/kinematics were determined with the Hamilton-Thorne IVOS analyser (Hamilton-Thorne Research, Beverly, MA) with standard set-up parameters. The analyser settings were as follows: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low average path velocity (VAP) cut-off, 5 m/s; low VSL cut-off, 11 m/s; head size, nonmotile, 3; head intensity, nonmotile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells not motile; magnification, 2.01, and temperature, 37°C.

Low sperm counts (<10 × 106/mL) were confirmed by manual analysis, while high counts (<50 × 106/mL) were diluted 1:1 with HTF medium for accurate determination of count by the CASA (IVOS) system.

3.2.8 Statistical Analyses

All statistical evaluations and tests were carried out using GraphPad Prism 2.01. Data are expressed as mean±SE. Student's *t*-test for paired data was used to compare the results of all the acrosome and motility studies, while Pearson's test was used to perform correlation tests. The Mann-Whitney *U* test for nonparametric data was used to compare binding results. A Hemizonae Index was also calculated for matched hemizonae assay results and expressed as a mean percentage where HZI = $^{\text{Test sperm bound to hemizona}}/_{\text{Control sperm bound to hemizona}}$ x 100). *P*-values equal or less than 0.05 were considered statistically significant.

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"There is one thing even more vital to science than intelligent methods; and that is, the sincere desire to find out the truth, whatever it may be."

- Charles Sanders Pierce -

CHAPTER 4

THE *ZONA PELLUCIDA-INDUCED* ACROSOME REACTION OF HUMAN SPERMATOZOA INVOLVES EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION

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Running head: Role for ERK during acrosome reaction

Keywords: spermatozoa, acrosome, ERK, *zona pellucida*

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Summary

Extracellular signal-regulated kinases (ERK), a member of the family of mitogen-activated protein kinases (MAPK) are cytoplasmic and nuclear serine/threonine kinases involved in signal transduction of several extracellular effectors. Recent evidence indicates the presence of p21Ras and the phosphorylation of ERK-1 and ERK-2, suggesting the occurrence of the Ras/ERK cascade in mammalian spermatozoa. We report here on the biological role of ERKs during the acrosome reaction, on stimulation with *zona pellucida* (ZP), in human spermatozoa. The mitogen-activated protein kinase inhibitor PD098059 was used as a pharmacological tool to study the involvement of extracellular signal-regulated kinases in the induction of the acrosome reaction in human spermatozoa. This compound significantly inhibited both the ZP- and A23187-induced acrosome reactions. These results suggest that ERKs are involved in the signal transduction pathway through which ZP stimulation works during the process of fertilization.

Introduction

The development of the fertilization-competent state of the spermatozoon occurs through a series of poorly understood processes including capacitation and acrosomal reaction (AR). During the process of capacitation spermatozoa acquire the ability to fertilize the oocyte. Capacitation is characterised by a series of profound membrane and metabolic transformations in spermatozoa that increase their ability to respond to physiological stimuli of the acrosome reaction (Yanagimachi, 1994) [1]. The AR is a specialised exocytotic event consisting of multiple fusion's and fenestration's of the outer acrosomal membrane and the overlying plasma membrane that lead to subsequent release of the acrosomal enzymes that help the spermatozoa

to penetrate the *zona pellucida* (Yanagimachi, 1994). These two activational processes appear to be regulated by intracellular signalling systems similar to those utilised by somatic cells (Kopf *et al.*, 1995). Although the precise signal transduction pathways have not been fully elucidated (Baldi *et al.*, 1996) it is known that both processes are characterised by increases in intracellular calcium concentrations and phosphorylation of proteins including that in tyrosine residues (Leyton & Saling, 1989; Carr & Acott, 1990; Naz *et al.*, 1991; Duncan & Fraser, 1993; Burks *et al.*, 1995; Luconi *et al.*, 1995; Visconti *et al.*, 1995; Luconi *et al.* 1996).

Several reports from studies in somatic cells focused attention on the presence and activation of the mitogen-activated protein kinase (MAPK) family of kinases in response to ligands binding both G-protein coupled receptors as well as protein tyrosine kinases (PTK) receptors. The MAPK's are a family of serine/threonine kinases with multiple membrane, cytosolic and nuclear substrates, the activation of which lead to an array of responses. This include the activation of (gene transcription via translocation of MAPK into the nucleus) genes encoding for protective proteins in response to stress, cell proliferation, cell differentiation, apoptosis and exocytosis (Offermans *et al.*, 1994; Page & Doubell, 1996; Canman & Kastan, 1996). It is also known that the activation of the MAPK family of kinases can be up- or down regulated through crosstalk with other signalling pathways i.e. protein kinase A (PKA), protein kinase C (PKC) and PTK in many cell types (Bogoyevitch *et al.*, 1994; Burgering & Bos, 1995). Extracellular signal-regulated kinases, ERK-1 (p44 MAPK) and ERK-2 (p42 MAPK), are the members of this family studied the most in somatic cells. The presence and biological activity of p21 Ras has recently been demonstrated in human spermatozoa (Naz *et al.*, 1992). Recent characterisation of a

boar sperm protein kinase with characteristics similar to those of ERK-2 (Berruti, 1994), as well as demonstrating the presence of ERK's in human spermatozoa (Luconi *et al.*, 1998) and the activation thereof during *in vitro* capacitation lead to the hypothesis that the Ras/Raf/ERK cascade may be involved in the capacitation and/or AR in human spermatozoa during *zona pellucida* (ZP) stimulation.

As of yet very little is known about ZP-mediated sperm signal transduction in the human, due, for the most part, to an inability to obtain sufficient quantities of human ZP for experimental purposes. The human ZP has been shown to bind human spermatozoa and to induce the AR of spermatozoa (Cross *et al.*, 1988; Morales *et al.*, 1989).

The present study aimed to determine the regulatory role of MAPK and in particular ERK during the acrosome reaction in human spermatozoa.

Materials and Methods

Preparation of sperm samples

Semen samples were collected from normozoospermic donors by masturbation after 2-3 days of sexual abstinence. Semen samples were analysed according to the World Health Organisation criteria (WHO, 1992) together with strict sperm morphology assessment (Kruger *et al.*, 1986). Motile sperm fractions were collected from samples using a slightly modified double-wash swim-up technique. Retrieved sperm samples were resuspended in synthetic human tubal fluid medium (HTF) (Quin *et al.*, 1985) supplemented with 3% bovine serum albumin (BSA; Seravac,

Cape Town, South Africa) to a sperm concentration of $10x10^6$ cells/ml. Before the onset of AR studies, sperm samples were allowed to capacitate at 37°C in 5% CO_2 for 3 hours in HTF-BSA. Prepared sperm samples were incubated in the presence or absence of the MEK-inhibitor, PD098059 (P-215, Sigma, Cape Town, South Africa) at a concentration of 50μM for 90 minutes at 37°C.

Preparation of solubilized ZP

Oocytes were retrieved from *post mortem* derived ovarian material. (The project fully complies with the legal and ethical guidelines of the South African Medical Research Council.) Oocytes were stored in a dimethylsulfoxide/sucrose solution at –196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25M sucrose and 3% BSA in HTF. On the day of the experiment, 20 oocytes were placed in a drop on a petri dish, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 20 oocytes on the petri dish. A total volume of 5μl of 10mM HCL was then added to the oocytes on the petri dish; solubilization of the ZP was microscopically observed and controlled. Following solubilization, 5μl of 10mM NaOH was added to the solubilized ZP, to render a final *zona* volume of 10μl containing 2 ZP/μl. The final ZP concentration, after the addition of spermatozoa, was 0.67 ZP/μl.

Acrosome reaction studies

Acrosomal statuses of spermatozoa stimulated with (i) 10μM A23187 (C-7522, Sigma, Cape Town, South Africa) for 20 minutes and (ii) 0.67ZP/μl for 60 minutes in either the presence or absence of PD098059 (pre-treatment) were determined

according to procedures published elsewhere (Cross *et al.*, 1988; Morales *et al.*, 1989, Franken *et al.*, 2000) and compared to that of control samples. Control samples were allowed to spontaneously acrosome react.

Prior to aspiration into Teflon tips during the micro-assay (Franken *et al.*, 2000), the sperm/ZP suspensions were gently mixed in a well of a 60 well micro-titre plate (Microtest plate cat No. P43, Laboratory and Scientific, Cape Town South Africa). Aspirating HTF droplets into both sides of the Teflon tip sealed off the sperm suspensions and prevented evaporation from the tip. Each sperm/ZP suspension was separated from the HTF droplets by air bubbles on both sides.

Progressive motility for both acrosome reaction techniques was monitored before and after the incubation periods. Sperm droplets were carefully placed on separate spots of spotted slides (MAGV, Germany, XER 201B) and immediately evaluated for percentage live sperm under inverted phase contrast microscope (Nikon TMS-F, Research Inst. Johannesburg, South Africa). Sperm samples were obtained after swim-up and only samples with a progressive motility of more than 80%, according to the World Health Organisation criteria (WHO, 1999), were subsequently used in the experiments.

Spermatozoa from the different experiments were fixed and air-dried, after which acrosomal status was determined using fluorescein-labeled *Pisum Sativum* agglutinin (FITC-PSA; 125μg/ml; L-0770; Sigma, Cape Town, South Africa). Two individuals using the blind scoring method scored a minimum of 200 spermatozoa for each different point. The following staining patterns were evaluated as acrosome reacted

spermatozoa; (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as an equatorial bar and (iii) no staining over the entire sperm surface. Spermatozoa with patchy FITC-PSA staining were classified as a population of sperm where the acrosome reaction was initiated and all were classified as acrosome reacted.

Statistical Analysis

The percentage acrosome reacted sperm were compared using Student's paired *t* test. *p*-values equal or less than 0.05 were considered statistically significant.

Results

Acrosome reactions induced spontaneously were completely insensitive to the pre-treatment of spermatozoa with PD098059 and the mean percentage of acrosome-reacted spermatozoa remained. As expected, A23187 stimulation induced the AR and the mean percentage of acrosome-reacted spermatozoa increased with 18.8%, which was significantly higher than that of the control values ($p<0.05$). However, after PD pre-treatment, A23187 stimulation did not induce the AR reaction significantly more than control values (Figure 1).

ZP stimulation also induced the AR and increased the mean percentage of acrosome reacted spermatozoa significantly by 9.2% ($p<0.05$). PD098059 pre-treatment of spermatozoa resulted in a complete inhibition of ZP stimulated AR and the mean percentage of acrosome reacted spermatozoa stayed at basal values (13.5%) (Figure 2).

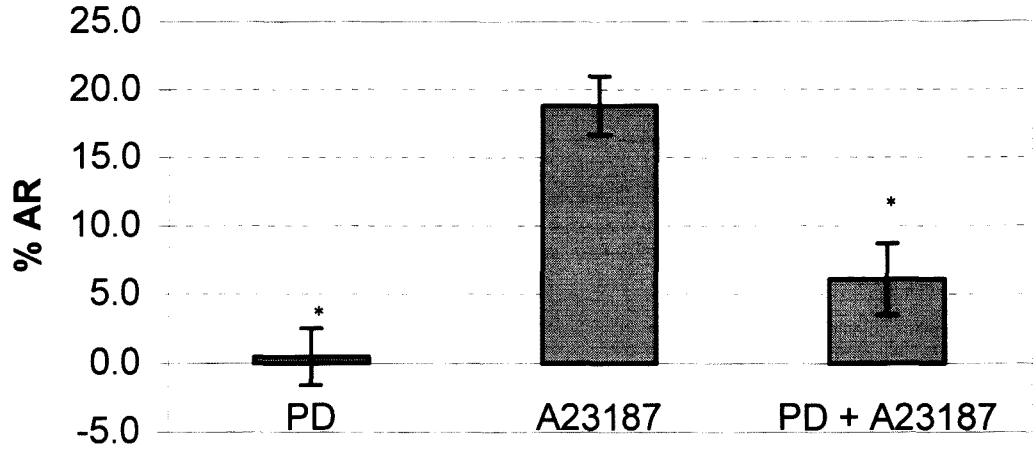

Figure 1. *Influence of the MEK-inhibitor PD098059 (PD) on the acrosome reaction (Mean±SE) mediated by A23187 (difference between acrosome reaction and the spontaneous percentage acrosome reaction of 23.9±2.2%). (n=10)*

** p<0.05 compared with A23187*

Capacitation seems to have been completed before the incubation of the spermatozoa with the MEK-inhibtor (PD098059) since these cells were able to acrosome react in response to both ZP and A23187 stimulation (results not shown).

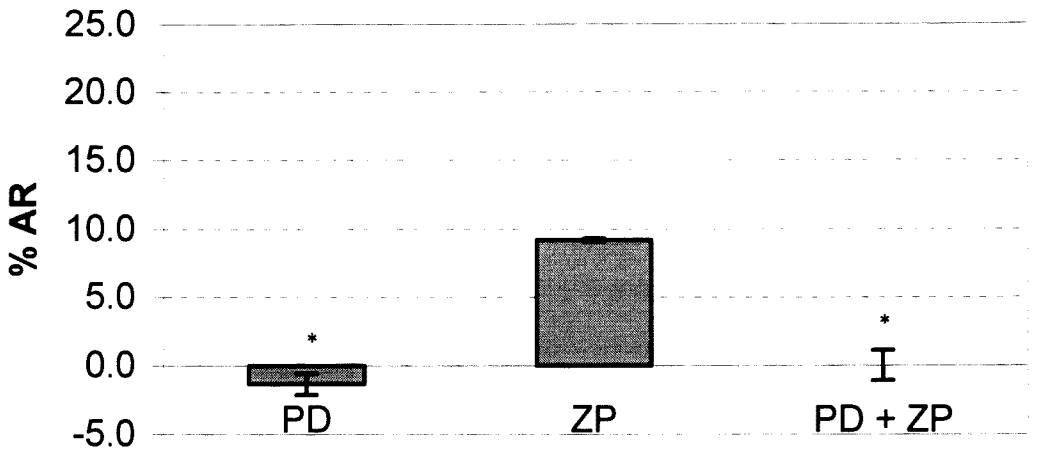

Figure 2. *Influence of the MEK-inhibitor PD098059 (PD) on the acrosome reaction (Mean±SE) mediated by ZP (difference between acrosome reaction and the spontaneous percentage acrosome reaction of 13.5±2.0%). (n=3)*

** p<0.001 compared with ZP*

Discussion

The present paper shows that ERK activation plays a biological role in the ability of human spermatozoa to undergo the acrosome reaction, since the ability of these cells to acrosome react in response to ZP and the calcium ionophore, A23187, is strongly inhibited in the presence of the MAPK (ERK) cascade inhibitor, PD098059.

To understand the signalling pathways through which both receptor induced (ZP3 binding to the ZP membrane receptor) and non-receptor induced (opening of calcium channels by an ionophore) mechanisms could elicit the acrosome reaction, a modified version of the recently hypothesized scheme of Breitbart and Spungin

(1997) will be referred to (Figure 3). The ZP3 glycoprotein binds to at least two receptors in the plasma membrane. One receptor is a G_i-coupled receptor that activates phospholipase C (PLC) ß1. The other receptor is a tyrosine kinase receptor (TKR) coupled to PLC_γ Binding to the G_i-coupled receptor would regulate adenylate cyclase (AC) leading to the elevation of cAMP and PKA activation. PKA activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane, which releases Ca^{2+} from the interior of the acrosome to the cytosol. This relative small rise in $[Ca^{2+}]_i$ could result in the activation of PLC_γ The products of phosphatidyl-inositol biphosphate (PIP_2) hydrolysis by $PLC_{ß1}$ and PLC_γ, diacylglycerol (DAG) and inositoltrisphosphate (IP_3) lead to PKC translocation to the plasma membrane and its subsequent activation. This increase in $[Ca^{2+}]_i$ can be mimicked by the addition of a calcium ionophore (e.g. A23187), which will also result in the activation of PLC_γ, and PKC activity. PKC opens a voltage-dependent Ca^{2+} channel in the plasma membrane, increasing the $[Ca^{2+}]_I$ even more. PKC activation also results in the activation of ERK, through its ability to phosphorylate and activate an upstream mediator of the ERK cascade, Raf. Raf will activate and phosphorylate the ERK kinase, MEK, which in turn activates ERK. The G_i and TKR can also activate a Na^+/H^+ exchanger, leading to alkalization (pH increase) of the cytosol. The increase in $[Ca^{2+}]_i$ and pH will lead to membrane fusion and acrosomal exocytosis.

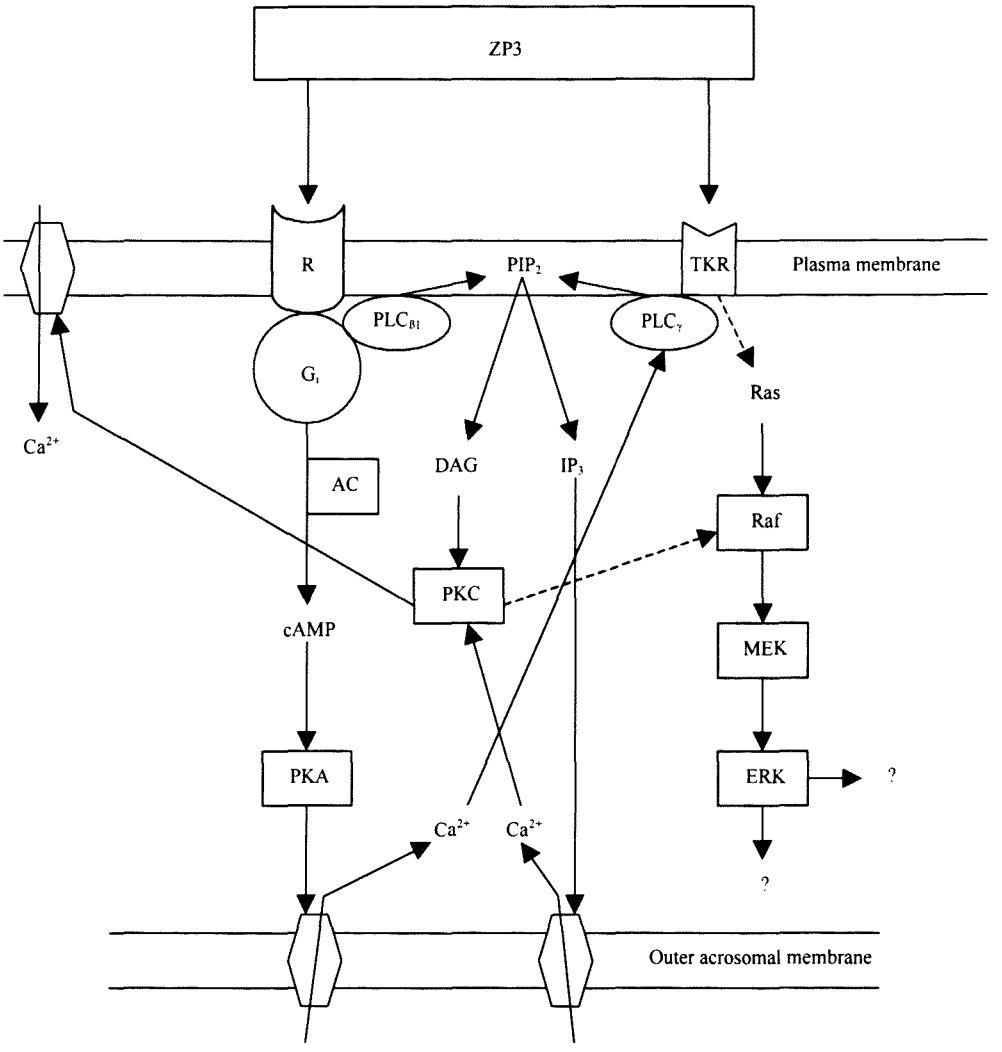

Figure 3. *Possible interactions between the different signal transduction pathways invoked during the acrosome reaction. (ZP3 = zona pellucida glycoprotein; R = G_i-coupled receptor; TKR = tyrosine kinase receptor; dashed lines = hypothesised activation of ERK) (Modified from Breitbart & Spungin, 1997)*

In support of this hypothesis, it was previously shown in our laboratories that the ZP-induced AR appears to be mediated through a G-protein-mediated signal transduction process after functional inactivation of the G_i-protein receptor by

pertussis toxin (Bastiaan *et al.*, 1999). Aitken *et al.* (1996) have also shown that the addition of the tyrosine kinase inhibitor, genistein, inhibited capacitation, probably by inhibiting the protein tyrosine kinase ZP receptor. In agreement with the hypothesis, Luconi *et al.* (1998) also showed that *in vitro* capacitation stimulates a sustained and concomitant increase in tyrosine phosphorylation and kinase activity of ERKs, indicating the activation of these enzymes during capacitation. It was also shown that the ability of human spermatozoa to undergo the AR in response to progesterone were strongly inhibited when capacitation was performed in the presence of the MAPK cascade inhibitor PD098059 (Luconi *et al.*, 1998).

The cellular targets of activated ERKs include, nuclear-, cytosolic-, cytoskeletal- and membrane proteins (Bokemeyer *et al.*, 1996, Gonzales *et al.*, 1993). In mitotic cells, the main target of activated ERKs, is the nucleus, where it phosphorylates transcriptional factors, ultimately resulting in *de novo* protein synthesis and cell proliferation. In non-mitotic mature spermatozoa the nucleus plays hardly, if any, a role in the modulation of their biological function and therefore a nucleur translocation of ERKs seem unlikely. Thus the presence of ERKs at the level of the equatorial segment after the AR (Luconi *et al.*, 1998) might be related to the regulation and/or activation of proteins that mediate binding and fusion between the sperm and egg plasma membranes. It has also been demonstrated that a conformational rearrangement of proteins, which appear to play an important role in the sperm-oocyte fusion at the level of the equatorial membrane, take place after stimulation of the AR (Allen & Green, 1995).

The downstream targets of ERKs during capacitation and the AR of spermatozoa remain to be defined. Seeing that both capacitation and AR are earmarked by the rearrangement of cytoskeletal elements, a possible target might be cytoskeletal elements such as microtubule-associated proteins (MAP), whose phosphorylation may be important in the cytoskeletal rearrangements that occur during capacitation (Duncan & Fraser, 1993).

In conclusion, our data demonstrates that ERKs are directly or indirectly involved in the acrosome reaction induced by human *zona pellucida*.

Acknowledgements

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"A scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die and a new generation grows up that is familiar with it."

- Maxwell Planck -

CHAPTER 5

EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION INVOLVED IN HUMAN SPERM-*ZONA PELLUCIDA* BINDING

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Running head: ERK involved in sperm-*zona* binding

Keywords: spermatozoa, acrosome, ERK, *zona pellucida*, hemizona

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Summary

In a previous study involving the inhibition of the mitogen-activated protein kinase (MAPK), extracellular signal regulated kinase (ERK), we found that the very specific MAPK Kinase (MEK) inhibitor, PD098059, inhibited the *zona pellucida* (ZP) induced acrosome reaction. Since an intact acrosome on the spermatozoa is a prerequisite to ensure tight binding to the ZP, we investigated the *zona* binding potential of spermatozoa after PD098059 treatment of sperm followed by exposure to solubilized human ZP and calcium ionophore (A23187). PD098059 treated spermatozoa exposed to solubilized ZP bound significantly more to the ZP as compared to control spermatozoa also exposed to solubilized ZP (26.5±3.7 vs. 13.8±2.8, $p<0.05$). No significant differences in binding to the ZP were observed between PD098059 treated and untreated sperm populations after A23187 exposure. These results can be interpreted to support the idea that the ZP-induced AR is the physiologically relevant exocytotic event since it is the ZP-induced AR, and not the spontaneous (culture medium) or A23187 induced AR, which appears to be mediated through an ERK-mediated signal transduction process.

Introduction

Before a spermatozoon can penetrate and fertilize the oocyte, it has to undergo various physiological processes i.e. capacitation (series of membrane and metabolic transformations), acrosome reaction (AR - fusion of the outer acrosomal membrane with the plasma membrane leading to exocytosis of enzymes and exposure of new membrane domains), as well as tight binding to the oocyte (species specific binding of spermatozoa to the *zona pellucida* - ZP). The human AR, a Ca^{2+}-dependent exocytotic event that is regulated by voltage-operated calcium ion channels located

in the plasma membrane of the sperm head, is a crucial early step in the fertilization process, and must be completed prior to fusion with oocytes (Babcock & Pfeiffer, 1987; Florman *et al.,* 1992; Florman, 1994; Arnoult *et al.,* 1996; Son *et al.*, 2000). Under physiological conditions, sperm voltage operated calcium channels are usually activated by contact with the ZP (Florman *et al.,* 1992; Arnoult *et al.,* 1996), while it can also be experimentally mediated by progesterone (Foresta *et al.,* 1993).

The mammalian ZP, and specifically glycoprotein 3 (ZP3), is the main mediator of sperm-egg recognition, sperm binding and the AR in mammals (Yanagimachi, 1994). ZP3 induces Ca^{2+} influx into cytoplasm, leading to increases of intracellular $Ca^{2+}$and pH, and resulting in acrosomal exocytosis (Babcock & Pfeiffer, 1987). The human ZP has been shown to bind human spermatozoa and to induce the AR of spermatozoa in both the intact and the solubilized state (Bielfield *et al.*, 1994; Cross *et al.*, 1988; Morales *et al.*, 1989). However, these methods cannot be used routinely in human studies, due to the scarcity of human material. Instead, several bovine serum albumin (BSA) neoglyco-proteins were shown to be able to stimulate the AR by interacting with the putative receptor for ZP3 in human spermatozoa (Brandelli *et al.*, 1996; Blackmore & Eisoldt, 1999).

Extracellular regulated kinase (ERK), a member of the serine/threonine family of mitogen activated kinases, can be phosphorylated and activated through both G-protein- and protein tyrosine kinase (PTK) receptor ligand binding in many cell types (Canman & Kastan, 1996; Page & Doubell, 1996). Both of these receptor types also play a role in the intracellular signalling systems regulating the processes of capacitation and AR. In support of this hypothesis, it was previously shown in our

laboratories that the ZP-induced AR appears to be mediated through a G-protein-mediated signal transduction process after functional inactivation of the G_i-protein receptor by pertussis toxin (Bastiaan *et al.*, 1999; Franken *et al.*, 1996). Aitken *et al.* (1996) have also shown that the addition of the tyrosine kinase inhibitor, genistein, inhibited capacitation, probably by inhibiting the protein tyrosine kinase ZP receptor. It has recently been shown that ERK's are present in human spermatozoa (Luconi *et al.*, 1998). We have also showed recently in our laboratories that the specific inhibition thereof significantly reduced the *ZP* stimulated *AR* in human spermatozoa (Du Plessis *et al.*, 2001).

The notion that ERK might play an important regulatory role in sperm *ZP* binding and thus sperm-oocyte binding was thus further pursued during this study.

Materials and Methods

Preparation of sperm samples

Semen samples were collected from normozoospermic donors by masturbation after 2-3 days of sexual abstinence. Semen samples were analysed according to the World Health Organisation criteria (WHO, 1999) together with strict sperm morphology assessment (Kruger *et al.*, 1986). Motile sperm fractions were collected from samples using a slightly modified double-wash swim-up technique. Retrieved sperm samples were resuspended in synthetic human tubule fluid culture medium (HTF) (Quin *et al.*, 1985) supplemented with 3% bovine serum albumin (BSA; Seravac, Cape Town, South Africa) to a sperm concentration of $10x10^6$ cells/ml. Before the onset of AR studies, sperm samples were allowed to capacitate at 37°C in

5% CO_2 for 3 hours in HTF-BSA. Prepared sperm samples were incubated in the presence or absence of the MEK-inhibitor, PD098059 (P-215, Sigma, Cape Town, South Africa) at a concentration of 50μM for 90 minutes at 37°C.

Preparation of solubilized ZP

Oocytes were retrieved from *post mortem* derived ovarian material. (The project fully complies with the legal and ethical guidelines of the South African Medical Research Council.) Oocytes were stored in a dimethylsulfoxide/sucrose solution at –196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25M sucrose and 3% BSA in HTF. On the day of the experiment, 20 oocytes were placed in a drop on a petri dish, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 20 oocytes on the petri dish. A total volume of 5μl of 10mM HCL was then added to the oocytes on the petri dish; solubilization of the ZP was microscopically observed and controlled. Following solubilization, 5μl of 10mM NaOH was added to the solubilized ZP, to render a final *zona* volume of 10μl containing 2 ZP/μl (Liu and Baker, 1996). The final ZP concentration, after the addition of spermatozoa, was 0.67 ZP/μl.

Hemi*zona* Assays/*Zona Pellucida* Binding

Spermatozoa were stimulated with (i) 10μM A23187 (C-7522, Sigma, Cape Town, South Africa) for 20 minutes and (ii) 0.67ZP/μl for 60 minutes in either the presence or absence of PD098059 (pre-treatment) to elicit the AR (Cross *et al.*, 1988; Morales

et al., 1989, Franken *et al.*, 2000) and compared to that of control samples. Control samples were allowed to spontaneously acrosome react.

Prior to aspiration into Teflon tips during the micro-assay (Franken *et al.*, 2000), the sperm/ZP and sperm/A23187 suspensions were gently mixed in a well of a 60 well micro-titre plate (Microtest plate cat No. P43, Laboratory and Scientific, Cape Town South Africa). Aspirating HTF droplets into both sides of the Teflon tip sealed off the sperm suspensions and prevented evaporation from the tip. Each sperm/ZP and sperm/A23187 suspension was separated from the HTF droplets by air bubbles on both sides.

Progressive motility of all samples was monitored before and after the incubation periods. Sperm droplets were carefully placed on separate spots of spotted slides (MAGV, Germany, XER 201B) and immediately evaluated for percentage live sperm under inverted phase contrast microscope (Nikon TMS-F, Research Inst. Johannesburg, South Africa). Only samples with a progressive motility of more than 80% were subsequently used in the experiments.

Both test and control sperm droplets (15μl; $10x10^6$sperm/ml) were placed on a petri dish to which hemi*zonae* were added in a match-controlled fashion. Hemi*zona* assays (HZA) were performed multi-fold and co-incubation was for four hours. Following the co-incubation period, hemi*zonae* were removed and washed (5x) to strip the loosely attached spermatozoa from the hemi*zonae*. Hemi*zonae* were then evaluated, while the number of spermatozoa tightly bound to the ZP was recorded for each test and matching control. Two individuals using the blind scoring method

scored the amount of spermatozoa bound for each hemi*zona* assay. Sperm-*zona* binding was also calculated for matched hemi*zonae* assay results and expressed as a mean percentage or Hemi*zona* Index (HZI) where HZI = $^{\text{Test sperm bound to hemizona}}/_{\text{Control sperm bound to hemi}zona} \times 100$.

Statistical Analysis

Sperm-*zona* binding results were expressed as the mean number of sperm bound per hemizona. The Mann-Whitney *U* test for nonparametric data was used to compare these binding results. *p*-values equal or less than 0.05 were considered statistically significant. A Hemi*zonae* Index was also calculated for matched hemi*zonae* assay results and expressed as a mean percentage. A HZI of 100% implies that the compound tested did not interfere with the amount of spermatozoa that bind to the ZP as compared to that of the control spermatozoa.

Results

Since the acrosome plays an important role during the binding and penetration of the ZP, the *zona*-binding capacity of PD098059 treated sperm populations was recorded. Table 1 illustrates the sperm-*zona* binding data. Results were obtained after PD098059 treatment of sperm followed by exposure to solubilized human ZP or calcium ionophore (A23187). Significantly more of the PD098059 treated spermatozoa bound to the ZP (mean±SE) as compared to control spermatozoa, after exposure to solubilized ZP (26.5±3.7 Vs. 13.8±2.8; $p<0.05$; Table 1). No significant differences in binding to the ZP were observed between PD098059 treated and untreated sperm populations after A23187 exposure.

Table 1. *Sperm-zona binding results after PD098059 treatment followed by exposure to Calcium Ionophore (A23187) and solubilized Zona pellucida (ZP).*

Control hemi*zona* assay		Test hemi*zona* assay	
Spermatozoa treated with different components alone		Spermatozoa exposed to components after PD098059 treatment	
Component	Mean (±SE) no. of zona-bound sperm	Component	Mean (±SE) no. of zona-bound sperm
Culture medium (n=15)	53.4±10.5[a]	Culture medium (n=12)	43.3±8.9
A23187 (n=12)	34.7±10.2	A23187 (n=12)	22.2±2.5
0.67ZP/μl (n=12)	13.8±2.8[b]	0.67ZP/μl (n=12)	26.5±3.7[c]

a Vs. b, $p<0.005$; b Vs. c, $p<0.05$

The results of the sperm *zona* binding of matched hemi*zonae* are expressed as a HZI in Table 2. It is evident that the MEK-inhibitor, PD098059, did not influence sperm-*zona* binding *per se* as a HZI of 107% was recorded when compared to the binding potential of control sperm (Table 2. *A*). A 14% increase in HZI was observed between PD098059-treated and untreated spermatozoa, after ZP stimulation prior to sperm-*zona* binding, when compared to that of their respective control hemi*zona* assays (Table 2. *D* = 28% Vs Table 2. *E* = 42%). PD098059 treated sperm populations exposed to solubilized ZP bound more to each hemi*zonae* as compared to spermatozoa incubated in a solution containing only ZP (HZI = 240%; Table 2. *G*). No real differences could be detected in sperm-*zona* binding between control and

PD098059-treated spermatozoa after A23187 stimulation when compared to that of their respective control hemi*zona* assays (Table 2. *B* = 57% Vs. Table 2. *C* = 54%).

Table 2. *Sperm-zona binding results expressed as a Hemizona Index (HZI) after PD098059 (PD) treatment followed by exposure to Calcium Ionophore (A23187) and solubilized Zona pellucida (ZP).*

#	Control hemi*zona* assay	Test hemi*zona* assay	HZI	n
A	Culture medium	PD + Culture medium	107%	5
B	Culture medium	A23187	57%	5
C	Culture medium	PD + A23187	54%	5
D	Culture medium	ZP	28%	7
E	Culture medium	PD + ZP	42%	5
F	A23187	PD + A23187	107%	4
G	ZP	PD + ZP	240%	5
H	PD + Culture medium	PD + ZP	41%	6
I	PD + Culture medium	PD + A23187	50%	5

$(HZI = {}^{Test}/_{Control} \times 100)$

Discussion

The present results illustrate the involvement of extracellular regulated kinase in the ZP stimulated *AR* (or physiological relevant exocytotic event) as opposed to the non-physiological A23187 induced event. The increase in binding of PD098059 pre-treated spermatozoa to the oocyte (13.8±2.8 Vs. 26.5±3.7 and HZI 28% Vs. 42%) is proof that the ZP induced *AR* was indeed inhibited and underline the possible regulatory effect of PD098059 and its subcellular targets on the ZP-induced AR.

PD098059 is a specific inhibitor of the activation of the ERK kinase (MEK), the kinase upstream of ERK, responsible for the dual phosphorylation and activation of ERK following G-protein coupled- and protein tyrosine kinase receptor ligation and activation, both *in vitro* and *in vivo* (Alessi *et al.*, 1995). Aitken *et al.* (1996) showed that the addition of genistein, a tyrosine kinase inhibitor, inhibited capacitation, probably by inhibiting the protein tyrosine kinase ZP receptor, while the functional inactivation of the G_i-protein receptor by pertussis toxin inhibited the ZP-induced AR which appear to be mediated through a G-protein mediated signal transduction process (Bastiaan *et al.*, 1999). Luconi *et al.* (1998) also showed that *in vitro* capacitation stimulates a sustained and concomitant increase in tyrosine phosphorylation and kinase activity of ERK; this activation of ERK was strongly inhibited when capacitation was performed in the presence of the MAPK cascade inhibitor PD098059 (Luconi *et al.*, 1998).

PD098059 also has effects seemingly unrelated to the inhibition of ERK, as shown by its functional inactivation of voltage-dependent calcium (Ca^{2+}) channels that participate in fertilization of the marine worm, *Urechis caupo* (Gould & Stephano, 2000). Voltage operated calcium channels can be classified into low voltage-activated and high voltage-activated calcium channels on the basis of thresholds for activation. High voltage-activated Ca^{2+} channels having high thresholds can be further classified by their pharmacological properties into L-, N-, P/Q-, and R-types (Birnbaurner *et al.,* 1994; Dunlap *et al.,* 1995). Widely used high voltage operated calcium channel antagonists can selectively block these channels. Stimulation of sperm with ZP depolarises the sperm membrane potential. ZP (specifically ZP3) stimulation activates a depolarisation mechanism with the characteristics of a poorly

selective cation channel. Pre-treatment of sperm with PD098059 possibly prevents activation of the Ca^{2+} -selective channel by ZP3/zonae *pellucidae* as is the case with pertussis toxin treatment (Florman *et al.*, 1995). The results can be interpreted to support the idea that the ZP-induced AR is the physiologically relevant exocytotic event since it is the ZP-induced AR, and not the spontaneous (culture medium) or A23187 induced AR, which appears to be mediated through a ERK-mediated signal transduction process.

Although it is generally accepted that the spermatozoa must be acrosome-reacted to complete penetration of the *ZP* (Franken *et al.*, 1991), the exact site of the AR has not been defined and appears to differ between species. PD098059 treatment of human spermatozoa does not affect the ability of spermatozoa to bind to structural intact human ZP. The results indicate the importance of intact acrosomes on the spermatozoa to ensure tight binding to the ZP, i.e., those sperm populations with a decreased AR; namely, the PD098059 treated spermatozoa bound significantly higher numbers of sperm during HZA conditions (Du Plessis *et al.*, *In press*).

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"Every great advance in science has issued from a new audacity of imagination."

- John Dewey -

CHAPTER 6

Phosphatidylinositol 3-kinase inhibition enhances human sperm motility and sperm-*zona pellucida* binding.

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Running head: PI3-Ks effect on sperm motility and binding

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Summary

Various signalling pathways are involved in the regulation of sperm motility, capacitation, acrosome reaction and sperm-*zona* binding. Recent data pointed out an important role for phosphatidylinositol 3-kinase (PI3K) in human sperm motility, however no studies as of yet has been done to determine the effect of this inhibitor on other sperm parameters. Here we investigated the role of PI3K on human sperm motility, acrosome reaction and sperm-oocyte binding by using the specific PI3K inhibitor LY294002. We demonstrate that *in vitro* incubation of washed unselected spermatozoa with LY294002, increased the percentage motility and progressive motility in asthenozoospermia patients as evaluated by computer-aided sperm analysis. The compound furthermore did not influence the acrosome reaction, whilst it further did enhance sperm-oocyte binding. Our results therefore imply that PI3K negatively affect sperm motility and oocyte binding. We subsequently suggest a possible therapeutic role for PI3K inhibitors in the treatment regime for asthenozoospermia.

Introduction

Spermatozoon prerequisites for initiation of fertilization include motility, capacitation and acrosome reaction. Normal motility patterns are needed to deliver the sperm to the site of fertilization, while concurrently it also has to undergo a series of functional biochemical and biophysical modifications named capacitation in order to render the ejaculated spermatozoa competent for fertilization of the oocyte. Finally a timeous acrosome reaction, which is an exocytotic process physiologically, induced by ligand (ZP3)-receptor interaction, ultimately lead to sperm-*zona* binding.

Many patients who attend infertility clinics are diagnosed with asthenozoospermia that result in impaired fertilization rates *in vivo* as well as during *in vitro* procedures (Oehninger, 2001). Even with the onset of advanced assisted reproductive techniques like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the quest to increase the fertilizing ability of semen samples is a very relevant and ongoing process. It is expected that simplified and more cost-efficient therapeutic modalities will be developed as additional basic (cellular-molecular) and clinical knowledge is gained (Edirisinghe *et al.*, 1995; Oehninger, 2001). It is therefore one of the goals of reproduction physiologists to try and increase the motility of spermatozoa among asthenozoospermic patients without interfering with the normal physiological processes needed for fertilization.

Various studies have demonstrated that several pharmacological agents such as SpermSolute (based on a proteinase – trypsin) and pentoxifylline can definitely improve sperm motility (Tesarik *et al.*, 1992; Krausz *et al.*, 1994; Lanzafame *et al.*, 1994; Minhas & Ripps, 1996; Figenschau & Bertheussen, 1999; Terriou *et al.*, 2000). However, the possibility of using such agents is limited due to the presence of non-responding subjects (Krausz *et al.*, 1994) and the possible toxic effects of these molecules (Centola *et al.*, 1995)

Even though it is known that phosphatidylinositol 3-kinase (PI3K) is present in mouse spermatozoa (Feng *et al.*, 1998), it was only recently demonstrated that LY294002, the specific inhibitor of PI3K, increased the progressive motility of spermatozoa in humans (Luconi *et al.,* 2001). Dolci *et al.* (2001) reported that stem cell factor (SCF) acts as a mitogenic factor in cultured c-kit-expressing spermatogonia and that both

mitogen-activated protein kinase kinase (MEK)- and PI3K-dependent pathways are required for the proliferative response. The mitogenic effect is not accompanied by an increase in total cellular amount of cyclin D3, but it is associated with a rapid change in its subcellular localisation. It was also shown that SCF is an anti-apoptotic factor for spermatogonia, but the MEK- or the PI3K-dependent pathways are not sufficient on their own to promote the survival response. It was further suggested that the binding of c-kit to SCF in mature sperm cells may result in the activation of PLCγ1 and PI3K, leading to receptor autophosphorylation and ultimately may play a role in capacitation and/or the acrosome reaction (Feng *et al.*, 1998).

PI3K's are SH2 domain-containing proteins, which is known to be important in phosphoinositide-mediated intracellular signalling pathways in numerous cell types. And is heterodymeric, consisting of a p85 regulatory (adapter) subunit and a p110 catalytic subunit. It phosphorylates the 3'-OH group of the inositol ring in inositol phospholipids and the most important of these is thought to be the conversion of phosphatidylinositol (PtdIns)-4,5-bisphosphate to PtdIns-3,4,5-trisphosphate (Fisher *et al.*, 1998). PI3K's have been identified in most cells studied. It has been implicated in the priming of a series of signalling cascades involved in the regulation of many processes of somatic cells including mitogenesis, differentiation, motility, cell survival, oocyte maturation, cell chemotaxis, membrane ruffling, DNA synthesis, receptor internalisation, vesicular trafficking and metabolic control (Kapeller & Cantley, 1994; Vanhaesebroeck *et al.*, 1997; Wymann & Pirola, 1998; Anderson *et al.*, 1999). It has also been suggested that PI3K might possibly be involved in the AR elicited by the mannose-BSA agonist by making use of the unrelated PI3K inhibitor, wortmannin (Fischer *et al.*, 1998).

As of yet no studies have been performed to test the effect of this inhibitor on the ZP induced AR as well as sperm-*zona* binding potential. The study aimed to investigate the role of PI3Ks in human sperm motility, AR and sperm-*zona* binding by using the PI3K inhibitor, LY294002.

Materials and Methods

Preparation of sperm samples

Semen samples were collected from volunteers (average age = 25.81±1.09 years; n=18) and analysed and classified according to the WHO criteria (WHO, 1999).

Only normozoospermic samples were used to perform AR and sperm-oocyte binding tests. Motile sperm fractions were collected after a double wash in synthetic human tubal fluid culture medium (HTF) (Quin *et al.*, 1985) and 60-minute swim-up technique in HTF supplemented with 3% bovine serum albumin (BSA; Seravac, Cape Town, South Africa). Retrieved sperm samples were resuspended in synthetic HTF-BSA to a sperm concentration of $10x10^6$ cells/ml. These fractions were then capacitated in the presence or absence of the PI3K-inhibitor, LY294002 (LY; 10µM; 120min; 37°C, 5% CO_2 and 95% humidity).

The semen samples used to perform computer-aided sperm analysis (CASA) underwent a double wash in HTF. The pellet containing the spermatozoa was then resuspended to its original volume in HTF + BSA. After preparation these samples

were incubated (37°C, 5% CO_2 and 95% humidity) for 2h in the presence or absence of the PI3K antagonist, LY294002 (10μM; 120min).

Preparation of solubilized ZP

Oocytes were retrieved from *post mortem* derived ovarian material. Approval was obtained from the Institutional Review Board and fully complies with the legal and ethical guidelines of the South African Medical Research Council. Retrieved oocytes were stored in a dimethylsulfoxide/sucrose solution at –196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25M sucrose and 3% BSA in HTF. On the day of the experiment, 20 oocytes were placed in a drop on a petri dish, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 20 oocytes on the petri dish. A total volume of 5μl of 10mM HCL was then added to the oocytes on the petri dish; solubilization of the ZP was microscopically observed and controlled. Following solubilization, 5μl of 10mM NaOH was added to the solubilized ZP, to render a final *zona* volume of 10μl containing 2 ZP/μl (Du Plessis, 2001). The final ZP concentration, after the addition of spermatozoa, was 0.67 ZP/μl.

Sperm kinematics

The samples prepared for motility studies were analysed using a Hamilton-Thorne (Hamilton-Thorne Research, Beverly, MA) IVOS analyser (integrated visual optical system) with standard set-up parameters. Washed spermatozoa were resuspended in a capacitation medium containing HTF with 3% BSA. Computer assisted semen analysis (CASA) was performed on each sample at 3 hours after collection using a

10.0μm chamber (Makler) and a sampling rate of 30 frames at 60Hz. A minimum of 100 cells and four fields was analysed for each aliquot. All analyses were performed at 37°C.

Acrosome reaction studies

Acrosomal statuses of spermatozoa stimulated with 0.67ZP/μl for 30 minutes in either the presence or absence of LY294002 (pre-treatment) were determined according to procedures published elsewhere (Cross *et al.*, 1988; Morales *et al.*, 1989, Franken *et al.*, 2000) and compared to that of control samples. These stimulation procedures were performed as micro-assays as previously described (Franken *et al.*, 2000).

Spermatozoa from the different experiments were fixed and air-dried, after which the acrosomal status was determined using fluorescein-labeled *Pisum Sativum* agglutinin (FITC-PSA; 125μg/ml; L-0770; Sigma, Cape Town, South Africa). Two individuals scored a minimum of 200 spermatozoa blindly for each different sample. The following staining patterns were evaluated as acrosome reacted spermatozoa: (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as an equatorial bar and (iii) no staining over the entire sperm surface.

Hemi*zona* Assays/*Zona Pellucida* Binding

Both LY294002 pre-treated (test) and control sperm samples (15μl droplets; $10x10^6$sperm/ml) were placed on a petri dish to which hemi*zonae* were added in a match-controlled fashion. Duplicate hemi*zona* assays (HZA) were performed and sperm-*zona* binding was assessed after four hours of co-incubation. Following the

co-incubation period, hemi*zonae* were removed and washed (5x) to strip the loosely attached spermatozoa. Hemi*zonae* were then evaluated, while the number of spermatozoa tightly bound to the ZP was recorded for each test and matching control. Experiments where less than 20 spermatozoa bound to a control hemi*zona* were discarded. Sperm-*zona* binding was expressed as a percentage or Hemi*zona* Index (HZI) where HZI = $^{\text{Test sperm bound to hemizona}}/_{\text{Control sperm bound to hemi}zona} \times 100$. A HZI of 100% would therefore imply that the compound tested did not interfere with the amount of spermatozoa that bind to the ZP as compared to that of the control spermatozoa.

Statistical Analysis

Sperm kinematics results, percentage acrosome reacted sperm and numbers of sperm bound to the hemi*zonae* are expressed as mean±SE. Student's *t*-test for paired data was used to compare the results of the motility, acrosome and hemi*zona* studies. Pearson's test was used for correlation tests when comparing different motility parameters. *P*-values equal or less than 0.05 were considered statistically significant.

Results

Sperm kinematics

Overall semen samples (n=18) showed a statistically significant increase in progressive motility after co-incubation with the PI3K inhibitor, LY294002, (31.88±3.75% vs. 37.31±3.74%, P=0.020). A complete shift in velocity distribution also occurred. According to CASA more of the static, slow and medium cells became

activated and the rapid population increased from 43.63±4.80% in the control samples to 50.38±4.20% in the LY294002 pre-treated samples (P=0.025). The rest of the sperm kinematics parameters did not change statistically significantly after LY294002 treatment in the donor population as a whole.

The results were subsequently divided into two groups (Table 1) based on the % motility of each donor's sperm sample. The groups were classified as >50% motility (normozoospermic) and <50% motility (asthenozoospermic) according to WHO (1999) criteria. The effect of PI3K inhibition is much more pronounced in the asthenozoospermic group compared to the normozoospermic group. The percentage motile and percentage progressive motile spermatozoa increased statistically significantly by 22% (from 29.25% to 51.25%; P=0.009) and 10.25% (from 15.00% to 25.25%; P=0.008) respectively after LY294002 treatment (Table 1). The velocity distribution results of this group also showed a statistically significant change with the rapid moving population increasing by 15.25% (from 21.50% to 36.75%; P=0.010) and the static population decreasing by 22% (from 70.75% to 48.75%; P=0.009).

An inverse effect occurred in the amplitude of lateral head displacement (ALH) between the two groups after LY294002 treatment. (The normozoospermic populations ALH increased from 4.23±0.17µm to 4.44±0.14µm (P=0.046) while the asthenozoospermic populations decreased from 5.00±0.43µm to 4.38±0.30µm (P=0.017)). When all of the control and LY pre-treated CASA results were pooled and analysed together a positive correlation (r=0.6487; P<0.0001; r^2=0.4208; n=36) was found between the progressive motility values and the beat cross frequency (BCF) values (Figure 1). No correlation existed between progressive motility and ALH, while

upon comparing BCF to ALH (Figure 2) a negative correlation was found (r=-0.3462; P=0.026; r^2=0.1199) in this group.

Table 1. *Sperm kinematics results of all the samples as well as when divided into normozoospermic and asthenozoospermic donors in the presence and absence of LY294002*

	All samples		>50% Motility		<50% Motility		*P*
	Control (n=18)	LY (n=18)	Control (n=12)	LY (n=12)	Control (n=6)	LY (n=6)	
Motile (%)	62.87±6.14	69.06±4.69	74.08±3.62	75.00±4.38	29.25±10.44 *	51.25±9.38 *	* 0.009
Progressive (%)	31.88±3.75 *	37.31±3.74 *	38.50±3.38	41.33±4.01	15.00±5.48 #	25.25±6.26 #	* 0.020 # 0.008
Path velocity (VAP, μm/s)	64.23±2.86	65.31±2.50	65.70±2.87	68.23±2.36	59.78±7.99	56.58±5.49	
Progressive velocity (VSL, μm/s)	54.85±3.09	55.54±2.59	56.71±2.90	58.39±2.28	49.25±9.14	47.00±6.75	
Track speed (VCL, μm/s)	100.89±4.07	105.09±2.88	101.36±4.84	106.84±3.28	99.48±8.54	99.83±5.91	
Lateral amplitude (ALH, μm)	4.43±0.18	4.43±0.12	4.23±0.17 *	4.44±0.14 *	5.00±0.43 #	4.38±0.30 #	* 0.046 # 0.017
Beat frequency (BCF, Hz)	26.20±0.97	27.01±1.08	27.17±1.09	27.08±1.25	23.48±1.64	26.80±2.43	
Straightness (STR, %)	82.56±146	82.81±1.31	83.50±0.99	83.50±1.01	79.75±5.31	80.75±4.60	
Linearity (LIN, %)	54.13±1.96	53.31±1.97	55.83±1.54	55.00±1.50	49.00±6.23	48.25±6.52	
Elongation (%)	69.44±0.94	68.00±0.63	68.33±0.51	67.67±0.75	72.75±3.12	69.00±1.08	
Area (μm sq)	4.35±0.15	4.16±0.12	4.42±0.16	4.16±0.14	4.15±0.41	4.18±0.25	
Rapid (%)	43.63±4.80 *	50.38±4.20 *	51.00±4.11	54.92±4.28	21.50±7.51 #	36.75±8.26 #	* 0.025 # 0.010
Static (%)	37.13±6.14	30.88±4.68	25.92±3.61	24.92±4.35	70.75±10.44 *	48.75±9.38 *	* 0.009

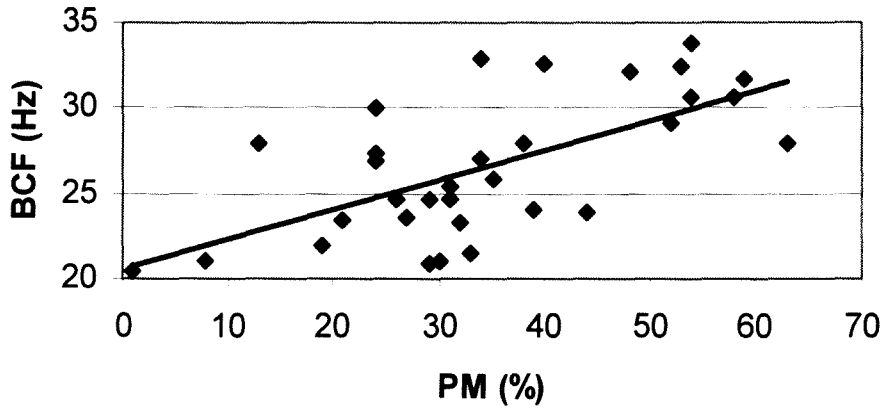

Figure 1. *Correlation between beat cross frequency (BCF) and progressive motility (PM) of pooled experiments (n=36). (r=0.6487; P<0.0001; r^2=0.4208)*

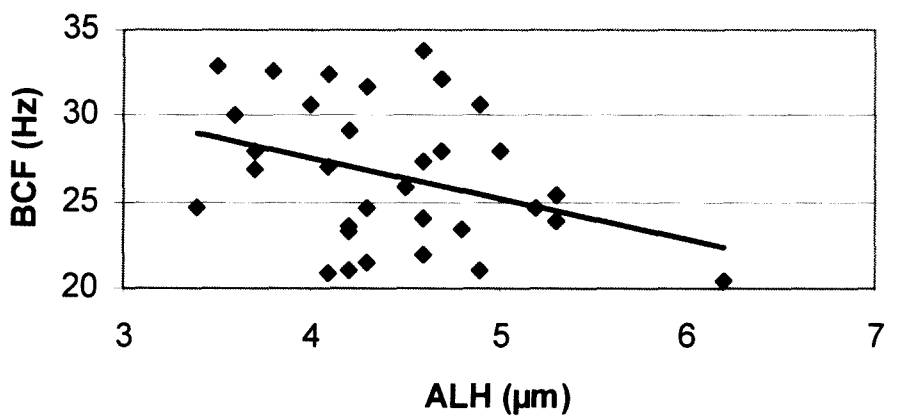

Figure 2. *Correlation between beat cross frequency (BCF) and amplitude of lateral head displacement (ALH) of pooled experiments (n=36). (-r=0.3462; P=0.026; r^2=0.199)*

Acrosome reaction

Due to the scarcity of human ZP, the effect of LY294002 on the spontaneous AR or the ZP-induced physiologically relevant exocytotic event was only determined in a limited number of normozoospermic sperm samples. ZP stimulation increased the %AR by a statistically significant 106.1% (P=0.017) in the control sample (from 10.18±1.924% to 20.98±1.805%), while a statistically significant increase of 112.3% was recorded in the LY pre-treated group (from 8.525±2.234 to 18.1±1.882). Though decreases in AR were detected in spontaneous (less 16.3%) and ZP induced AR (less 13.7%) after LY pre-treatment, it was not of significant value. It can thus be deducted that exposure of spermatozoa to the PI3K inhibitor, LY294002, did not influence the spontaneous or ZP induced acrosome reactions during these experiments (n=5; Figure 3).

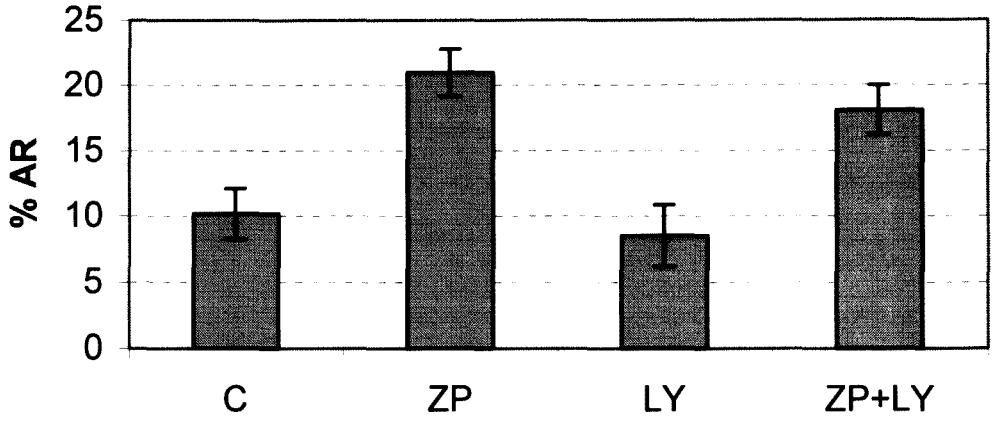

Figure 3. *Histogram showing the percentage acrosome reaction (mean±SE) of control (C) spermatozoa and spermatozoa after exposure to zona pellucida (ZP), PI3K antagonist LY294002 (LY) or both ZP and LY294002 (ZP+LY). LY did not inhibit or increase the spontaneous or ZP induced acrosome reactions (n=5). [C vs. ZP (P=0.017); C vs. LY (P=0.608); C vs. ZP+LY (P=0.007), ZP vs. LY (P=0.009); ZP vs. ZP+LY (P=0.323); LY vs. ZP+LY (P=0.019)]*

Sperm-*zona pellucida* binding

A statistically significant difference (P=0.024) could be detected between the number of LY294002 pre-treated spermatozoa (49.72±2.7; range 29.0-80.0 and median = 49.5) and control spermatozoa (45.11±2.6; range 28.0-70.0 and median = 44.0) that bind to the hemi*zonae*'s respectively (Figure 4). The ratio between LY pre-treated (test) and untreated (control) samples produced a HZI of 112% ± 5%. This suggests a trend that more LY pre-treated spermatozoa did bind to the hemizona.

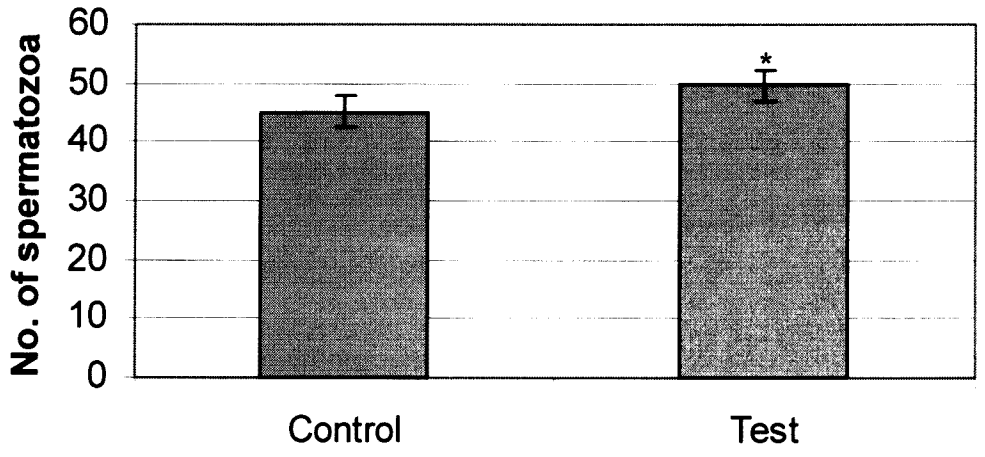

Figure 4. *Histogram showing the number of control and LY294002 pre-treated (Test) spermatozoa (mean±SE) tightly bound to each hemizona respectively (n=18). (* P=0.024 vs. control)*

Discussion

Controversies do exist regarding sperm motility enhancers and their effects on sperm viability and fertilizing ability. In the present study it was demonstrated that the addition of LY294002, a very specific inhibitor of phosphatidylinositol 3-kinase, is able

to increase the motility and progressive motility of human spermatozoa. These results are strongly supported by similar studies performed by Luconi *et al.* (2001). We specifically show that the addition of LY294002, to double washed sperm samples, increase progressive motility and the percentage rapid cells in our general donor population. In addition we demonstrate that our results are even more exaggerated in the asthenozoospermic population. Not only did the progressive motility and rapid cell population increase dramatically, but also the percentage motile cells increased while percentage static cells decreased markedly. Luconi *et al.* (2001) also showed increases in VCL, VAP and VSL (and other motion parameters) as measured by CASA. We however did not find any significant increases in these parameters but did see increasing trends in these parameters among all our sperm populations. Not finding statistic significance in this study regarding the above mentioned parameters can be ascribe to making use of smaller sample populations as well as differences in preparational techniques (e.g. centrifuged, washed, unselected sperm vs. swim-up selected sperm). The molecular mechanisms underlying the stimulatory effects of LY294002 on sperm motility are still poorly understood. The process of development and maintaining of motility in mammalian spermatozoa is rather complex and involves the integration of and crosstalk of several signalling pathways, including adenylate cyclase/cAMP/PKA, calcium and phosphorylation/dephosphorylation of proteins (Tash and Bracho, 1994). Luconi *et al.*, (2001) speculate that in spermatozoa PI3K might be involve in phosphorylating or dephosphorylating proteins involved in motility or in the generation of reactive oxygen species (ROS) which has a negative impact on motility.

It can also be deducted from our results that BCF is closely related to progressive motility and thereby an increase in BCF might lead to an increase in progressive

motility. It was also shown that BCF and ALH correlates negatively. It can thus be further deducted that in order for BCF to increase, there are less time for the sperm head to be displaced sideways and thereby decreasing ALH.

Knowing that the PI3K signalling pathway is involved in eliciting of the AR (Fisher *et al.*, 1998), it was therefore important to investigate the effects of LY on this reaction. In our hands LY294002 did not affect the spontaneous or the ZP induced AR even though a slight decline in AR was observed in all 5 experiments between the ZP and the LY+ZP groups. Furthermore no significant (P=0.163) difference in AR was observed between progesterone stimulated spermatozoa with or without LY treatment (results not shown). Luconi (personal communication) also found that LY exerts no effect on both the spontaneous and progesterone stimulated AR, thereby supporting the current data. Contrary to our findings, Fischer *et al.* (1998) showed a decrease in the AR elicited by the mannose-BSA agonist by making use of the unrelated PI3K inhibitor, wortmannin. Yet wortmannin was found not to inhibit the acrosome reaction induced by either A23187 or progesterone (Fischer *et al.,* 1998). This may imply that the cellular pathways involved in bringing about the acrosome reaction induced by the different agonists (ZP3, Progesterone, Mannose-BSA, A23187) do not involve PI3K to the same extend, or perhaps that the need for PI3K in the pathway is somehow bypassed. It may also imply that LY294002 and wortmannin have different mechanisms of action or differ in their specificity to inhibit PI3K. In this regard wortmannin has indeed been shown to inhibit other enzymatic activities (Cross *et al.*, 1995) including MAPK and extracellular signal regulated kinases (ERK) at doses from 250nM (Ferby *et al.*, 1996). The presence of ERK has been demonstrated in human spermatozoa (Luconi *et al.*, 1998) and implicated to be involved in the ZP-induced AR (Du Plessis *et al.*, 2001). It can therefore be

speculated that the decrease in AR in wortmannin treated samples after exposure to the mannose-BSA agonist may not only be due to PI3K inhibition, but also due to the possible simultaneous inhibition of ERK. However as half-maximal inhibition of MAPK activation occurs at 300nM this must be ruled out as Fisher *et al.* (1998) used the inhibitor at concentrations ranging between 10nM and 100nM.

When considering that the different agonists (i.e. ZP3, Progesterone, Mannose-BSA) all stimulate different signalling pathways through binding to different membrane receptors, it is obvious that it will result in different cellular responses. From the literature it is evident that various crosstalk mechanisms are involved in PI3K and RAS/RAF/ERK signalling. It is known that RAS can both activate RAF/ERK as well as PI3K (Sears & Nevins, 2002; Krasilnikov, 2000). PI3K on the other hand can also activate the RAS/RAF/ERK pathway through mitogen signalling (Krasilnikov, 2000) (see Figure 5). Taking into consideration that mannose-BSA stimulation elicit the AR through both G_i-protein receptor binding (Brandelli *et al.*, 1996) and receptor tyrosine kinase binding (Fisher *et al.*, 1998), and that PI3K is regulated by receptor tyrosine kinase activity, it can explain why PI3K inhibition can inhibit the mannose-BSA induced AR (Fisher *et al.,* 1998). Wortmannin will inhibit PI3K, thereby also inhibiting the PI3K activated RAS/RAF/ERK pathway needed for eliciting of the AR (Du Plessis, 2001). There is no evidence in the literature that the mannose-BSA tyrosine kinase receptor is directly linked to the RAS/RAF/ERK pathway, but only through PI3K. In our experiments we stimulated the AR with solubilized ZP (ZP3), thereby eliciting the AR through both a G_i-protein receptor and ZRK binding which directly activates RAS/RAF/ERK and PI3K through RAS (see Figure 5). Inhibition of PI3K by LY294002 thus only inhibited the PI3K pathway and not the direct RAS/RAF/ERK pathway activated independent of PI3K signalling or activation through protein kinase

C (PKC). The activation of RAS by ZP binding to ZRK can also stimulate PI3K, thereby enhancing a possible positive feedback on ERK activation (e.g. via PKC) as well as other mediators in the process of AR signalling (see Figure 5).

A statistically significant increase in sperm-oocyte binding after LY treatment was also detected. It was also previously shown in our laboratory that the frequency of sperm/*zona pellucida* collision rates has an influence on *zona* binding (Ozgür & Franken, 1996). This increase in binding can therefore be ascribed to the fact that more spermatozoa are motile and progressively motile, thereby increasing the chances of them colliding with the oocyte. Myles and Primakoff (personal communication) showed similar results in the mouse model by finding that incubation of mouse sperm and oocyte in the presence of LY significantly increases sperm-*zona* binding.

Compared with signalling pathways in many types of somatic cells, the signal transduction pathways of the mammalian acrosome reaction (*pellucida* glycoprotein) are still very poorly understood (Fisher *et al.*, 1998). One of the chief reasons for this is that the sperm receptor(s) that bind to *zona* protein 3 (ZP3) and physiologically induce the acrosome reaction remain to be conclusively identified (Brewis & Moore, 1997). Indeed, in the human, about ten sperm determinants in spermatozoa have been reported to be involved (listed and referenced in Benoff, 1997). The most likely scenario is that several sperm determinants are involved in *zona* binding and function in concert as part of a complex (Kopf *et al.*, 1995; Brewis & Moore, 1997).

Our results imply that PI3K negatively regulates sperm motility and increase sperm-*zona* binding without influencing the acrosome reaction. This suggests that

LY294002 can be used as a tool to enhance the motility of sperm samples during preparation for IVF from patients with low sperm motility, thereby ultimately opening a new prospective for severe oligoasthenozoospermic males to enter IVF rather than ICSI programs.

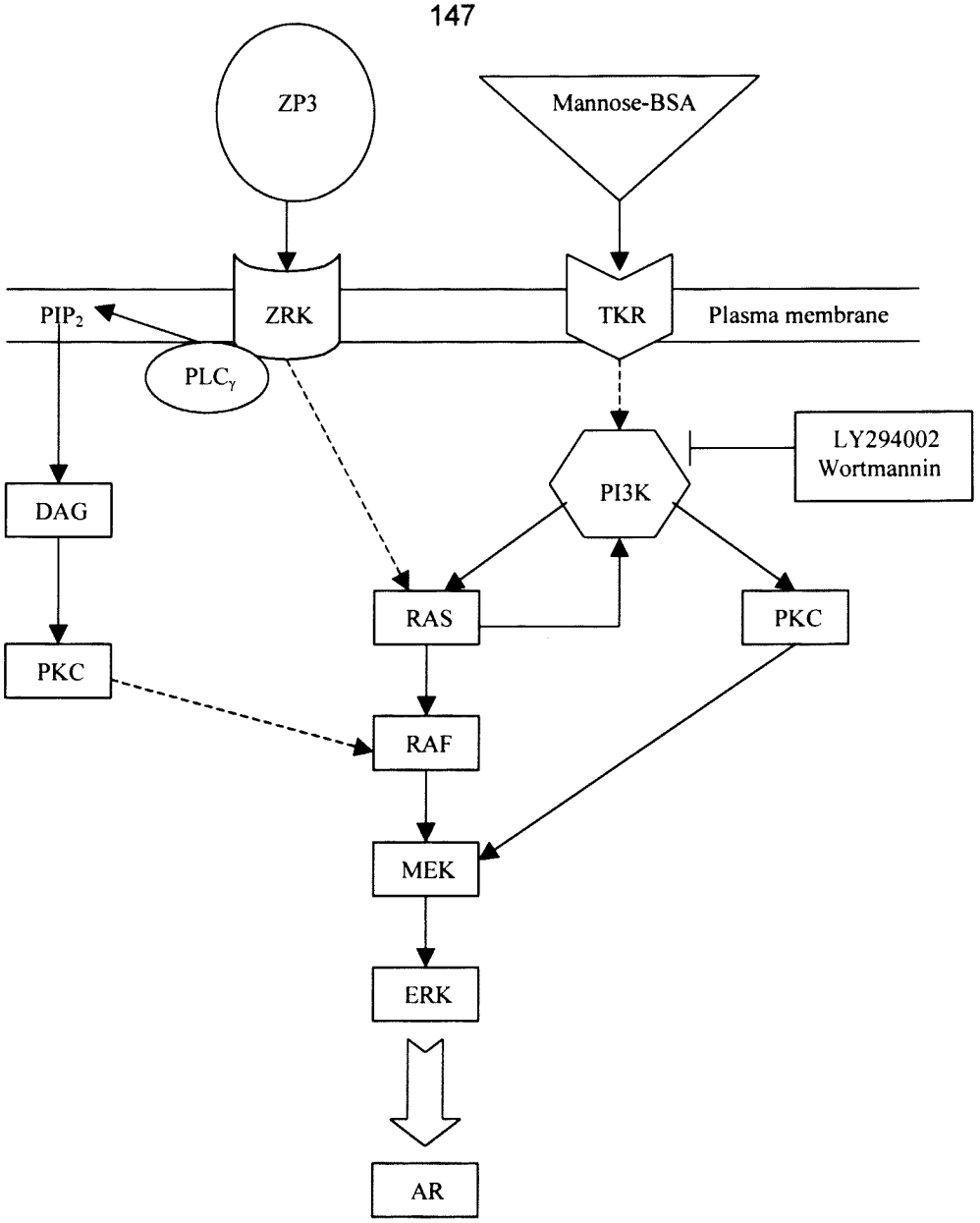

Figure 5. *Possible interactions between the different signal transduction pathways invoked during the acrosome reaction. (ZP3 = zona pellucida glycoprotein 3; ZRK = Zona Receptor Kinase; TKR = mannose tyrosine kinase receptor; dashed lines = hypothesised activation of ERK; G_i-coupled receptor pathway not shown)*

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The erection is the last gasp of modern manhood. If men can't continue to produce erections, they're going to evolve themselves right out of the human species.

- Time Magazine, 5 May 1998 -

CHAPTER 7

The effect of acute *in vivo* sildenafil citrate (VIAGRA™) treatment on semen parameters and sperm function

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Running head: Effect of sildenafil citrate on sperm parameters and function

Keywords: sildenafil citrate, spermatozoa, acrosome, *zona pellucida*, motility

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Summary

Viagra™ is a powerful treatment for male erectile dysfunction of various aetiologies. Sildenafil citrate, the active ingredient of Viagra™, is a specific and potent inhibitor of phosphodiesterase type 5 and thus enhances the activity of the nitric oxide-cGMP pathway that promotes penile erection due to cGMP accumulation. In this study we investigated whether acute *in vivo* sildenafil citrate (50mg orally) administration modifies seminal parameters, induction of the acrosome reaction, sperm-oocyte binding and sperm motility. No changes in the macro- and microscopical seminal parameters were caused by sildenafil citrate when compared to placebo. Sildenafil citrate also did not initiate or potentiate the acrosome reaction. Sperm-oocyte binding, smooth path velocity, straight line velocity and the percentage rapid cells all increased after sildenafil citrate treatment. These results suggest a clinical usefulness for sildenafil citrate in the enhancing of fertilizing ability of inherently poor quality sperm or for treatment during assisted reproductive techniques.

Introduction

Erectile dysfunction (ED) is a widespread condition that has a negative impact on quality of life, affecting both older and younger men in an estimated 10% of the adult population (Feldman *et al.*, 1994). Until recently no effective oral therapy existed and available treatments were highly cumbersome or invasive (Purvis *et al.*, 2002). Sildenafil citrate (Viagra™, Pfizer), a cyclic nucleotide phosphodiesterase (PDE)-inhibitor, was the first oral agent to be successfully introduced for the management of ED in which there is no established organic cause (Boolell *et al.*, 1996b). When administered before sexual activity, it produces reliable efficacy, good tolerability and rapid absorption that yields prompt onset of action (Boolell *et al.*, 1996a).

Cyclic nucleotide phosphodiesterases regulate intracellular levels of cyclic 3',5' adenosine monophosphate (cAMP) and cyclic 3',5' guanosine monophosphate (cGMP) by hydrolysing them to the corresponding 5' monophosphates (Conti *et al.*, 1995). Cyclic nucleotides serve as second messengers for a wide variety of extracellular signals such as nitric oxide, neurotransmitters, hormones, light and odourants. As of yet nine different PDE iso-enzymes (PDE1 to PDE9) have been described and found to be present at various concentrations in human tissues (Soderling *et al.*, 1998a; Fabbri *et al.*, 1999).

Nitric oxide synthase, a cGMP inducer (Lewis *et al.*, 1996), and two distinct PDE isoforms (PDE1 and PDE4) are present in human sperm cells (Fisch *et al.*, 1998). It was also shown in previous studies that mRNA coding for cAMP-specific PDE (PDE4A) isoforms are present in mature rat and mouse germ cells (Naro *et al.*, 1996) and that the expression of these isoforms is maximal in round spermatids and is maintained in mature spermatozoa (Soderling *et al.*, 1998b).

Phosphodiesterase type-5 (PDE5) is the predominant PDE iso-enzyme for the degradation of cGMP in the corpus cavernosum. Sildenafil citrate (Viagra™) is a specific and potent inhibitor of this cGMP-specific PDE5 enzyme and thus enhances the activity of the nitric oxide-cGMP pathway (Figure 1) that promotes penile erection due to cGMP accumulation (Ballard *et al.*, 1998; Glossman *et al.*, 1999). It also has minor inhibitory effects on PDE6 and PDE1 activities (IC_{50} = 0.034 and 0.28μmol/l respectively) (Morales *et al.*, 1998). Sildenafil citrate mimics cGMP and interacts with the catalytic site of PDE. During normal erections sexual stimulation lead to an increase in nitric oxide production which in turn stimulate cGMP production. These increased cGMP levels causes trabecular smooth muscle relaxation, cavernosal

artery dilatation, increased cavernosal pressure and penile erection. Sildenafil citrate potently enhances the relaxant effect of nitric oxide on the human corpus cavernosum by increasing the value of cGMP in this tissue. However sexual stimulation still remain mandatory in order to produce its pharmacological effect, since it has no direct relaxant effect on the corpus cavernosum (Fabbri *et al.*, 1997; Ballard *et al.*, 1998; Goldstein *et al.*, 1998). Viagra™ is rapidly absorbed and acts within 30 minutes and reach maximum plasma concentrations within approximately 1 hour. The absolute bioavailability is 41% due to first-pass metabolism and is rapidly cleared from the body with a plasma elimination half-life of 3-4 hours (Nichols *et al.*, 2002).

It is likely that men with ED will use sildenafil citrate as an aid to procreation, especially by younger patients who have ED e.g. secondary to spinal cord injury. It is also known that a degree of ED may be present in the male infertile partner especially when assisted reproductive techniques are necessary. These men have difficulties in producing spermatozoa on demand at the time of egg fertilization. Tur-Kaspa *et al.* (1999) showed that these forms of temporary erectile dysfunction can be successfully treated with sildenafil citrate. In this context however, the local affects of sildenafil citrate on sperm function have not been researched extensively.

A large number of drugs can be transported into the seminal fluid, where they have direct effects on function, physiology, metabolism or genetic composition of spermatozoa (Pichini *et al.*, 1994). Several drugs have been shown to affect sperm motility in particular; these include compounds with PDE–inhibitory activity that have the potential to increase motility (Schoenfeld *et al.*, 1975; Turner *et al.*, 1978). One such drug, pentoxifylline, which is a cAMP PDE inhibitor, has been shown to

stimulate human sperm motility both *in vitro* and *in vivo* (Shen *et al.*, 1991). It has also been demonstrated that human sperm cells contain as yet uncharacterised PDE isoforms which are different from PDE1 and PDE4, and that the *in vitro* inhibition of sperm PDE1 and PDE4 iso-enzymes by specific inhibitors stimulates acrosome reaction (AR) and sperm motility (Fisch *et al.*, 1998). It is also known that after acute oral administration of 100mg-sildenafil citrate, the drug reaches a concentration of 0.1-0.3 µmol/l in the ejaculate (Pfizer, Viagra™ data sheet). This concentration is consistent with possible inhibitory actions of sildenafil citrate on sperm PDE isoforms (Fabbri *et al.*, 1999).

Sildenafil citrate is rapidly becoming the treatment of choice for ED of various aetiologies even though many of its potential effects are still being investigated (Edwards and Muirhead, 2002). The drug is relatively lipophilic ($logD_{7.4}$=2.7) and would therefore be expected to distribute into the seminal fluid (Purvis *et al.*, 2002) where it subsequently might have important local effects/actions which still remain unknown. We therefore conducted a study to investigate and evaluate the effects of an acute single oral dose of sildenafil citrate (50mg) on a number of seminal, sperm functional and morphology parameters in young healthy male volunteers.

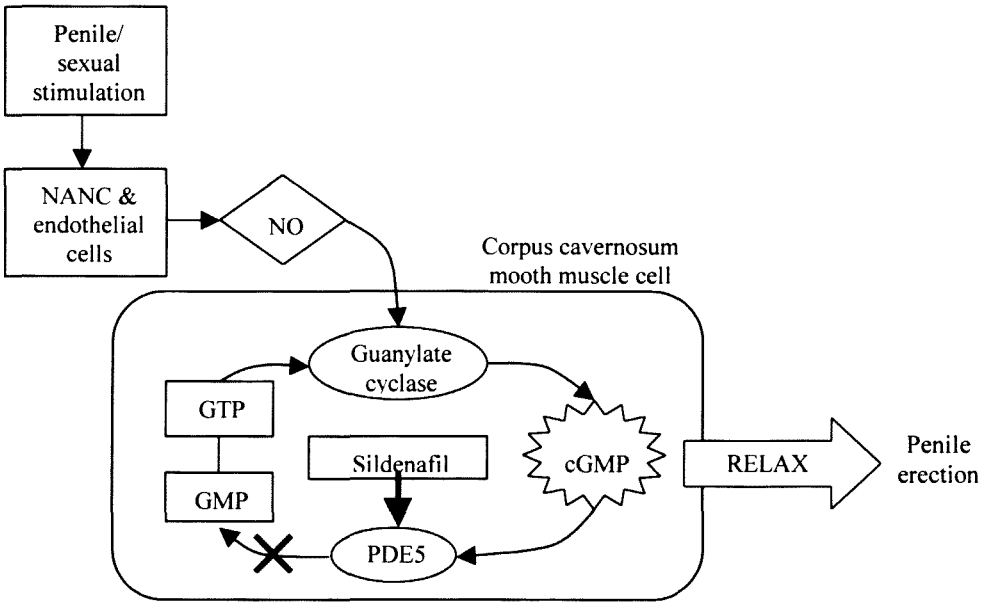

Figure 1. *Enhancement of the NO-cGMP mechanism of penile erection by sildenafil citrate. Inhibition by sildenafil citrate results in elevation of cGMP concentrations in the corpus cavernosum, which induces relaxation of corpus cavernosal smooth muscle, vasodilatation, increased blood flow to the penis, increased intracavernosal pressure and penile erection. NO = nitric oxide; cGMP = cyclic guanosine monophosphate; PDE5 = phosphodiesterase type 5; NANC = noradrenergic-noncholinergic neurones; GTP = guanosine triphosphate.*

Materials and Methods

Study design and subjects

The study design consisted of a prospective double blind, placebo-controlled, crossover, two period clinical investigation. The study blind was broken after completion of all investigations. All subjects enrolled spontaneously in the study after giving an informed consent to the study protocol, which was approved by the Ethical Committee of our institution. The inclusion criteria included normal erectile function, normal electrocardiogram, no prior or concomitant serious illness or consumption of medications during the 3-month period prior to the study. In a double-blind fashion, subjects randomly received one of the two treatments, that is 50mg sildenafil citrate (Viagra^{TM}, Pfizer, Sandton, RSA) or placebo to ingest orally. All subjects were asked not to ejaculate for a minimum of 3 days before each dose of study drug. They were not permitted to consume products containing alcohol, caffeine or methylxanthines or cigarette smoking for a period of three days before each session of the study. The subjects were also required not to use prescription or over-the-counter drugs during the course of the study. All study medications were taken under supervision with 50ml of water. The washout period consisted of a 7-day period in which no medication was given. After this time, all subjects were crossed over to receive the alternative treatment.

Written informed consent was obtained from each subject before study entry. Subjects who met the inclusion criteria underwent a pre-study screening in the 3 weeks before the start of the study that consisted of a physical examination,

measurement of supine blood pressure and pulse rate, a 12-lead electrocardiogram and a urine test.

An *in vitro* study was also conducted in parallel in order to investigate the effects of increased levels of exogenous cGMP on sperm motility, AR and sperm-oocyte binding. 8-bromo-cGMP (8-Br-cGMP), a non-hydrolyzable cGMP analogue, was used to increase intracellular cGMP levels *in vitro*, as it is lipid soluble and therefore able to enter the cell quite easily (in contrast to cGMP that cannot penetrate the cell membrane). The drug dissolved easily in water and was used at a final concentration of 20µM (60minutes).

Semen processing / Preparation of sperm samples

Semen samples were collected 1 hour after ingestion of the test drug by means of masturbation according to the World Health Organisation guidelines (WHO, 1999). Motile sperm fractions were collected from samples using a slightly modified double-wash swim-up technique. Retrieved sperm samples were resuspended in synthetic human tubal fluid culture medium (HTF) (Quin *et al.*, 1985) supplemented with 3% bovine serum albumin (BSA; Seravac, Cape Town, South Africa) to a sperm concentration of $10x10^6$ cells/ml. Before the onset of AR and sperm-oocyte binding studies, sperm samples were allowed to capacitate at 37°C in 5% CO_2 for 3 hours in HTF-BSA. Excess spermatozoa that were not used for motility or AR studies were cryopreserved (using SpermFreeze; FertiPro N.V, Beernem, Belgium) in 0.5 mL straws using a slow-freeze method (Mahedevan and Trounson, 1993) in order to perform matched hemi*zona* assays at a later stage.

Macro and microscopical seminal parameters

Ejaculate volume was measured volumetrically and pH was determined with Multistix Reagent Strips (Bayer Corporation, Tarrytown, NY, USA). Liquefication, viscosity and appearance of each sample were judged visually. Sperm concentration, sperm count and sperm head size (area) was acquired by means of computer assisted sperm analysis (CASA, see later). An assessment of morphology was made after staining with eosin Y and Harris haemotoxylin and spermatozoa was evaluated according to World Health Organisation guidelines (WHO, 1999) together with strict sperm morphology criteria (Kruger *et al.*, 1986).

Motility studies

Sperm kinematics was assessed after a 30-minute liquefication period at room temperature by means of Computer assisted semen analysis (CASA). The analysis was performed on an Integrated Visual Optical System for sperm analysis (IVOS, Hamilton Thorne Research, Beverly, MA) with standard set-up parameters. The raw semen was diluted 1:1 in HTF-BSA and placed in a disposable fixed-depth slide. (20.0μm, Makler) at an image capture rate of a 30 frames at 60Hz. A minimum of 200 cells and three fields was analysed for each aliquot. All analyses were performed at 37°C. The following measurements were performed:

Counts:

Total, Motile, Progressive

% Motile, % Progressively Motile

Rapid, Medium, Slow and Static Cells

Concentrations

Total, Motile, Progressive (millions/ml)

Rapid, Medium, Slow and static Cells (millions/ml)

Mean Values

VAP: Smoothed Path Velocity (microns/sec)

VCL: Track Velocity (microns/sec)

VSL: Straight Line Velocity (microns/sec)

ALH: Amplitude of Lateral Head Displacement (microns)

BCF: Beat Cross Frequency (hertz)

LIN: Linearity (ratio of VSL/VCL)

STR: Straightness (ratio of VSL/VAP)

Area: Head size (square microns)

Preparation of solubilized ZP

Oocytes were retrieved from *post mortem* derived ovarian material. (The project fully complies with the legal and ethical guidelines of the South African Medical Research Council.) Oocytes were stored in a dimethylsulfoxide/sucrose solution at –196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25M sucrose and 3% BSA in HTF. On the day of the experiment, 20 oocytes were placed in a drop on a petri dish, after which the HTF was removed under microscopic vision (Olympus SZ40; Wirsam Scientific, Cape Town, South Africa), leaving only the 20 oocytes on the petri dish. A total volume of 5µl of 10mM HCL was then added to the oocytes on the petri dish; solubilization of the ZP was microscopically observed and controlled. Following solubilization, 5µl of 10mM NaOH was added to the solubilized ZP, to render a final *zona* volume of 10µl containing 2 ZP/µl. The final ZP concentration, after the addition of spermatozoa, was 0.67 ZP/µl.

Acrosome reaction studies

Spermatozoa from both the Viagra or placebo groups were challenged with (i) 10μM calcium ionophore A23187 (C-7522, Sigma, Cape Town, South Africa) for 30 minutes, (ii) 1μg/ml progesterone for 30 minutes, (iii) 0.67ZP/μl for 30 minutes or (iv) left to spontaneously elicit the AR. The acrosomal status of these spermatozoa were subsequently determined according to procedures published elsewhere (Cross *et al.*, 1988; Morales *et al.*, 1989, Franken *et al.*, 2000).

Spermatozoa from the different experiments were fixed and air-dried, after which the acrosomal status was determined using fluorescein-labeled *Pisum Sativum* agglutinin (FITC-PSA; 125μg/ml; L-0770; Sigma, Cape Town, South Africa). Two individuals scored a minimum of 200 spermatozoa blindly for each different sample. The following staining patterns were evaluated as acrosome reacted spermatozoa: (i) patchy staining on acrosomal region, (ii) distinct staining in the equatorial region occurring as an equatorial bar and (iii) no staining over the entire sperm surface.

Hemi*zona* Assays/*Zona Pellucida* Binding

Straws containing the cryopreserved spermatozoa were thawed rapidly at 37°C for 10 minutes. Cryoprotectant was removed with the dropwise addition of P1 medium followed by centrifugation at 600 *g* for 10 minutes and resuspension in 0.5ml fresh medium (Mahedevan & Trounson, 1993). Both test (sildenafil citrate) and control (placebo) sperm droplets (15μl; $10x10^6$sperm/ml) were placed on a petri dish to which hemi*zonae* were added in a match-controlled fashion. Hemi*zona* assays (HZA) were performed in duplicate and co-incubation was for four hours. Following the co-incubation period, hemi*zonae* were removed and washed (5x) to strip the loosely attached spermatozoa from the hemi*zonae*. Hemi*zonae* were then evaluated by two

individuals using the blind scoring method, while the number of spermatozoa tightly bound to the ZP was recorded for each test and matching control. Sperm-*zona* binding was also expressed as a percentage or a Hemi*zona* Index (HZI) where the HZI = $^{\text{Test sperm bound to hemizona}}/_{\text{Control sperm bound to hemi}zona}$ x 100. A HZI of 100% would therefore imply that the compound tested did not interfere with the amount of spermatozoa that bind to the ZP as compared to that of the control spermatozoa.

Statistical Analysis

All results are expressed as the mean ± the standard error. Data were compared using Student's *t*-test for paired data. P-values equal or less than 0.05 were considered statistically significant. All statistical analysis were performed using GraphPad PRISM version 2.01.

Results

Demographic characteristics, safety and tolerability

Twenty healthy male volunteers were enrolled in the study. The subjects were all Caucasian and between 20 and 33 years of age (23.182 ± 0.689). No significant medical conditions relevant to the study were noted before entry or during the study. Adverse events considered by the investigator to be related to treatment occurred in 6 of 20 subjects (30%) following administration of sildenafil citrate and in 1 of 20 subjects (5%) following administration of placebo (Table I). The most frequently reported adverse events were flushing, headache and abnormal vision (blue vision) and were generally mild to moderate in severity. No subject discontinued treatment due to adverse effects related to sildenafil citrate.

Table I: *Incidence of adverse events following treatment with placebo or sildenafil citrate (n=20).*

	Placebo	Sildenafil 50mg
Treatment related adverse events	1/20	6/20
Flushing	0	3
Headache	0	2
Abnormal vision	0	2
Spontaneous erections	1	1

Macro- and microscopical seminal parameters

Table II shows the macroscopical seminal parameters after acute *in vivo* sildenafil citrate or placebo administration. Liquification, appearance and viscosity of the semen samples from both groups were normal as according to the WHO criteria. Mean values of semen volume (2.533±0.320ml vs. 2.373±0.313ml) and pH (8.182±0.076 vs. 8.318±0.076) did not show significant variations between the two treatment groups.

Table II: *Initial macroscopic appearance and evaluation of semen after either placebo or sildenafil citrate administration (n=20).*

Semen parameters	Placebo	Sildenafil 50mg	*P*
Liquefication	<60 minutes	<60 minutes	-
Appearance	Normal	Normal	-
Viscosity	Normal	Normal	-
Ejaculate Volume (ml)	2.533 ± 0.320	2.373 ± 0.313	0.432
pH	8.182 ± 0.076	8.318 ± 0.076	0.096

Similarly sildenafil citrate was equivalent to placebo in its effects on all microscopical secondary semen analysis parameters, including sperm count, sperm concentration and morphology (Table III). All these variables were also within the normal ranges as predefined by the study centre. The area of the sperm head size (square microns), as determined by CASA, was not affected by sildenafil citrate.

Table III: *Effects of acute in vivo administration of sildenafil citrate on microscopical secondary semen analysis parameters (n=20).*

Semen parameters	Normal values	Placebo	Sildenafil 50mg	*P*
Sperm count ($x10^6$)	100-500	220.8 ± 45.892	232.2 ± 54.453	0.449
Sperm concentration ($x10^6$/ml)	50-150	73.72 ± 13.32	77.52 ± 13.29	0.627
Morphology (% normal forms)	>15	15.059 ± 0.961	14.765 ± 0.893	0.281
Area (µm sq)		3.400 ± 0.228	3.350 ± 0.394	0.872

Acrosome reaction

From Table IV it is evident that the addition of exogenous cGMP did not affect the AR between treatment groups (control vs. 8-Br-cGMP) when left to spontaneously undergo the AR or when stimulated with A23187 or progesterone. The AR is thus independent of treatment with 8-Br-cGMP at a concentration of 20µM.

The results of the various AR studies from the clinical trial are displayed in Figure 2. When left to spontaneously undergo the AR, the placebo group showed a 13.94 ±

2.542% AR while the sildenafil citrate group showed no statistical significant difference (14.06 ± 2.128%; P = 0.753) in AR. It can be seen that after calcium ionophore (A23187) stimulation the percentage AR in both groups was statistically significantly elevated (Placebo: 23.37 ± 2.831%, P=0.0002; Sildenafil: 26.37 ± 3.598%, P=0.0001). Progesterone stimulation had a similar effect and also increased acrosome reactions in both groups with significant margins to above that of the spontaneous levels (Placebo: 21.21 ± 3.306%, P=0.004; Sildenafil: 24.90 ± 3.788%, P=0.007). Exposure to solubilized *zona pellucida* also increased the acrosome reactions significantly to above that of spontaneous levels in both groups (Placebo: 25.64 ± 5.797%, P=0.026; Sildenafil: 26.17 ± 3.898%, P = 0.014). The acrosome reactions between treatment groups (placebo vs. sildenafil) did not differ significantly after A23187 (P=0.893), progesterone (P=0.728) and *zona pellucida* (P=0.957) stimulation and were thus independent of treatment.

Table IV: *The effect of in vitro intracellular cGMP elevation by the addition of 8-Br-cGMP (20μM) on eliciting of the acrosome reaction (n=6).*

Stimuli	Control	8-Br-cGMP	P
Spontaneous	16.500±3.085	17.167±3.911	0.444
A23187	23.667±3.273	23.000±6.611	0.287
Progesterone	21.333±3.896	20.857±2.852	0.225

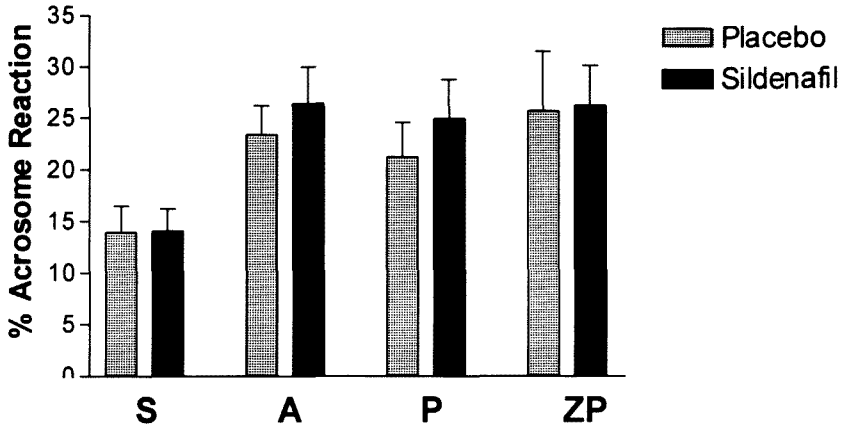

Figure 2: *The effects of double-blind placebo or 50mg-sildenafil citrate administration on the sperm acrosome reaction (n=20).*

S = spontaneous, A = A23187, P = progesterone, ZP = zona pellucida.

Placebo: S vs. A = 0.0002; S vs. P = 0.004; S vs. ZP = 0.026

Sildenafil: S vs. A = 0.0001; S vs. P = 0.0073; S vs. ZP = 0.0147

Sperm-*zona pellucida* binding

Incubation of spermatozoa in the presence of 8-Br-cGMP lead to increased sperm-*zona* binding and a HZI of 134% was determined (n=6, in duplicate). This percentage indicates that more spermatozoa bound to their respective matched hemi*zonaes* after exposure to 8-Br-cGMP (20µM).

The number of spermatozoa that bound to each matched hemi*zona* (Figure 3) was not statistically significantly influenced by acute *in vivo* sildenafil citrate administration even though a hemi*zona* index of 148.75% was recorded. These results suggest that a nearly 49% higher binding rate occurred after acute sildenafil citrate ingestion.

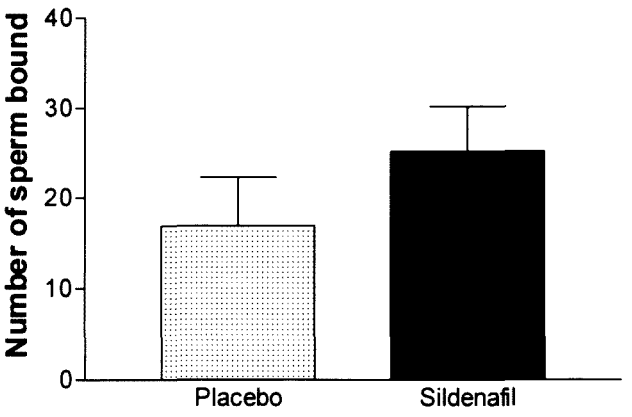

Figure 3: *The effect of double-blind placebo or 50mg-sildenafil citrate administration on the average number of spermatozoa tightly/firmly bound to each hemizona (n=10; P=0.281).*

Sperm kinematics results

On average more than 800 cells (817.8±71.92), but not less than 200 cells were evaluated in order to determine the various sperm motility parameters. The mean values for the sperm motility parameters were all within the normal ranges as predefined by the study centre (Table V). No significant difference was observed between placebo and sildenafil citrate for percentage motile, percentage progressive motile and percentage static cells. VCL, ALH, BCF, STR and LIN also did not differ between treatments. It was however found that both VAP (58.61±2.258μm.s^{-1} vs. 64.11±2.195μm.s^{-1}, P=0.047) and VSL (47.28±2.250μm.s^{-1} vs. 51.92±2.249μm.s^{-1}, P=0.046) increased statistically significantly after sildenafil citrate treatment. Furthermore it was found that after sildenafil citrate treatment a shift occurred from the medium, slow and static cell populations towards the rapid cell population thereby

increasing the percentage rapid cells significantly (44.47±4.782% vs. 50.88±5.180, P=0.009).

The percentage motile (61.0 ±3.488 vs. 82.00±2.415; P=0.036; n=6) percentage progressive motile (27.33±2.926 vs. 35.50±4.180; P=0.026; n=6) and percentage rapid (35.00±3.479 vs. 45.67±4.344; P=0.014; n=6) spermatozoa also increased significantly after incubation of spermatozoa in the presence of 8-Br-cGMP (20μM, 60minutes).

Table V: *Effects of 8-Br-cGMP (in vitro) and sildenafil (in vivo) on different sperm motility parameters as measured by CASA.*

Parameter	Normal values	Control (n=6)	8-Br-cGMP (n=6)	*P*	Placebo (n=20)	Sildenafil (n=20)	*P*
Motile (%)	>50	61.00 ± 3.488	82.00 ± 2.451	0.037	67.39 ± 5.892	68.22 ± 5.673	0.850
Progressive motility (%)	>15	27.33 ± 2.927	35.50 ± 4.180	0.027	30.89 ± 3.809	33.00 ± 3.801	0.530
Smoothed Path Velocity (VAP - µm/s)		54.96 ± 3.256	58.58 ± 4.898	0.190	58.61 ± 2.528	64.11 ± 2.195	0.047
Straight Line Velocity (VSL - µm/s)	>25	49.28 ± 3.261	51.93 ± 4.869	0.265	47.28 ± 2.250	51.92 ± 2.249	0.046
Track Velocity (VCL - µm/s)		72.38 ± 4.325	75.98 ± 4.606	0.288	90.18 ± 3.407	94.64 ± 4.009	0.324
Amplitude of Lateral Head Displacement (ALH - µm)		3.10 ± 0.202	3.00 ± 0.089	0.542	3.929 ± 0.1350	4.194 ± 0.2190	0.202
Beat Cross Frequency (BCF - Hz)		24.12 ± 1.492	24.03 ± 0.674	0.949	24.11 ± 0.8050	24.59 ± 1.136	0.656
Straightness (STR - %)		86.50 ± 1.440	85.67 ± 1.291	0.497	77.20 ± 1.377	78.88 ± 1.252	0.447
Linearity (LIN - %)		64.83 ± 2.518	65.67 ± 2.620	0.662	53.61 ± 1.684	54.61 ± 1.589	0.621
Rapid (%)	>25	35.00 ± 3.479	45.67 ± 4.344	0.014	44.47 ± 4.782	50.88 ± 5.180	0.009
Static (%)	<50	32.33 ± 7.298	22.667 ± 6.547	0.272	37.24 ± 6.822	32.35 ± 7.014	0.239

Discussion

A total of 20 male volunteers were enrolled in this prospective double blind, placebo-controlled, crossover, two period clinical investigation to determine the pharmacodynamic effects of sildenafil citrate on sperm function and ejaculate quality. Sildenafil citrate was well tolerated by the volunteers, producing no serious adverse events or clinically important changes in vital signs. The most frequently reported adverse events were generally consistent with those reported in other studies (Purvis *et al.*, 2002; Boolell *et al.*, 1996; Goldstein *et al.*, 1998; Morales *et al.*, 1998).

In this study a comparison of acute sildenafil citrate treatment vs. placebo showed no effect on a wide variety of macroscopical seminal measures including liquefication, appearance, viscosity, ejaculate volume or pH. Sperm count, sperm concentration, morphology and area of sperm head size was also not influenced. These results are in line with those reported in similar studies (Aversa *et al.*, 2000).

It was shown that exogenous cGMP did not initiate or potentiate the AR of capacitated spermatozoa, yet it is well known from the literature that cGMP serve as a signal transducer mediating the AR. When this signalling pathway is activated by using 8-Br-cGMP, effects are only documented at concentrations of higher than 0.5mM with peak activity at 1mM (Rotem *et al.*, 1998; Revelli *et al.*, 2001). This is most likely due to PKA stimulation because cGMP can interact with the regulatory subunit of PKA when used at concentrations higher than 100µM (Lincoln & Cornwell, 1993). In our experiments we used a concentration of 20µM, which did not result in the initiation of the AR. Capacitated spermatozoa also underwent an AR when

challenged with calcium ionophore, progesterone and *zona pellucida* plus in combination with the PDE inhibitor sildenafil citrate, but not with sildenafil citrate alone. These data suggest that sildenafil citrate (50mg orally) by itself cannot initiate the AR nor can it potentiate the AR of capacitated spermatozoa, as it most probably cannot elevate intracellular cGMP levels high enough through its inhibition of PDE5. Furthermore, Lefievre *et al.* (2000) similarly showed that PDE inhibitors by themselves had no effect on the AR.

Fisch *et al.* (1998) showed by means of immunocytochemical staining that different types of PDE are located at different sites in the spermatozoon e.g. PDE4 localises mainly to the mid-piece, while PDE1 is found more prominently in the sperm head. These observations support the idea that differential PDE localisation within the spermatozoon can allow for selective modulation of sperm function through the regulation of distinct pools of cAMP (Fisch *et al.*, 1998).

Sildenafil citrate is a controversial member of a drug class (PDE inhibitors) previously shown to either have some effects on sperm motility (Schoenfeld *et al.*, 1975; Turner *et al.*, 1978; Lefiévre *et al.*, 2000) or no effects on motility at all (Purvis *et al.*, 2000; Aversa *et al.*, 2000).

In this study a comparison of sildenafil citrate vs. placebo on a wide variety of kinematics parameters showed no effect on percentage sperm motility, percentage progressive motility, track velocity, amplitude of lateral head displacement, beat cross frequency, straightness, linearity or percentage static cells. Borderline statistical significant changes were however observed in smoothed path velocity and straight-

line velocity after sildenafil citrate administration. The most significant change of note was observed in the increase in percentage rapid cells after sildenafil citrate administration. Elevation of intracellular cGMP levels by 8-Br-cGMP also increased percentage motile, percentage progressively motile and percentage rapid cells significantly. Both Cuadra *et al.* (2000) and Lefievre *et al.* (2000) showed that the inhibition of PDE5 through the addition of sildenafil citrate also enhanced certain motility parameters. Cuadra *et al.* (2000) found an increase in sperm progressive motility and hyperactivation, while Lefievre *et al.* (2000) showed increases in VCL, ALH and hyperactivation.

The hemi*zona* indexes of 148 % after sildenafil citrate and 134% after 8-Br-cGMP administration refer to increases in sperm-oocyte binding. These increases in binding can possibly be explained by the fact that, combined with various increases in different motion characteristics, more spermatozoa are rapidly motile thereby increasing the chances of them colliding with the oocyte. This theory fits in with results previously obtained in our laboratory, that the frequency of sperm/*zona pellucida* collision rates has an influence on *zona* binding (Ozgür & Franken, 1996). This increase in hemi*zona* index is further proof that sildenafil citrate, and therefore PDE inhibition, does not elicit the AR as only AR intact cells can bind to the oocyte.

Lefiévre *et al.* (2000) discovered that the inhibition of sperm PDE by sildenafil citrate was associated with a significant increase in cAMP and that sperm PDE activity measured with cGMP as substrate was threefold lower than when measured with cAMP. There was also a dose-dependent increase in cAMP levels in spermatozoa incubated with sildenafil citrate (Lefiévre *et al.*, 2000). It was demonstrated that

spermatozoa contain PDE that can hydrolyse both cAMP and cGMP (Lefiévre *et al.*, 2000). However, whereas cAMP has often been linked to sperm function, the role of cGMP in sperm functions remains still unclear. The intracellular mechanism responsible for the sildenafil-induced cAMP increase in human spermatozoa is unknown, but it can be hypothesised that sildenafil citrate could act on PDEs other than type 5 (Lefiévre *et al.*, 2000). Sildenafil citrate induced similar changes in sperm motility parameters as nonselective PDE inhibitors such as pentoxifylline (Calogero *et al.*, 1998; Nassar *et al.*, 1999) caffeine (Cheng & Boettcher, 1981; Leclerc *et al.*, 1996) and IBMX (Leclerc *et al.*, 1996). These observations also suggest that sildenafil citrate probably acts on types 1 and 4 PDE because cAMP has been implicated in the regulation of sperm motility (Calogero *et al.*, 1998) through activation of PKA (Yanagimachi, 1994). It was also shown by Lefiévre *et al.* (2000) that sildenafil citrate increased capacitation and the associated tyrosine phosphorylation of p105/81, 2 fibrous sheath proteins. These 2 latter processes are recognised to be cAMP-dependent (Leclerc *et al.*, 1996).

In conclusion, inhibition of PDE by sildenafil citrate produces an increase in sperm kinematics parameters as well as sperm-oocyte binding, but no macroscopical or microscopical changes occurred. It also suggests that PDE is not strongly involved in the events leading to the human sperm AR.

Although the incidence of ED increases with age, younger men are also affected (Feldman *et al.*, 1994). As such, it is very likely that a significant number of patients will use sildenafil citrate as an aid to procreation. When studying erectile dysfunction drugs the focus was always on obtaining sexual satisfaction and never about sperm

quality. The results of this study clearly indicate that single oral doses of sildenafil citrate (50mg) can be safely administered without concern on the part of the physician or the patient regarding adverse effects on sperm or ejaculate quality. Furthermore, the ability of sildenafil citrate to enhance sperm kinematics results and sperm-oocyte binding without affecting the AR may be clinically useful. These findings could have important implications in the use of sildenafil citrate in assisted reproductive technology to enhance fertilizing potential of inherently poor quality sperm with less invasive treatments such as intrauterine inseminations, thereby avoiding more involved procedures such as IVF or ICSI.

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"Today, if you are not confused, you are just not thinking clearly."

- U. Peter -

CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusion

The physiological acrosome reaction occurs upon interaction of the spermatozoon with the *zona pellucida* protein ZP3 and is of fundamental importance in the fertilization of the oocyte by the spermatozoon. This is followed by the liberation of several acrosomal enzymes and other constituents that facilitate penetration of the *zona* and expose molecules on the sperm equatorial segment that allow fusion of the sperm membrane with the oolemma. The molecular mechanisms and the signal transduction pathways mediating the processes of motility, capacitation and the acrosome reaction have been partially defined and appear to involve modifications of intracellular calcium and other ions, lipid transfer and phospholipid remodelling in the sperm plasma membrane as well as changes in protein phoshorylation. However there are still many unanswered questions regarding human sperm interaction with the oocyte.

8.1.1 Motility

From the results obtained in this study it was evident that:

i) Phosphatidylinositol 3-kinase negatively regulates sperm motility.

ii) Inhibition of phosphodiesterase type 5 by sildenafil citrate, and therefore a possible accumulation of cGMP in the spermatozoon, lead to an increase in certain sperm kinematics parameters.

The process of development and maintaining of motility in mammalian spermatozoa is rather complex and involves the integration and crosstalk of several signalling pathways, including adenylate cyclase/cAMP/PKA, calcium and phosphorylation/dephosphorylation of various substrate proteins (Tash and Bracho, 1994).

The molecular mechanisms underlying the stimulatory effects of the PI3K inhibitor, LY294002, on sperm motility are still poorly understood. At this stage we can only speculate that in spermatozoa, PI3K might be involved in the phosphorylating or dephosphorylating of proteins involved in motility. Another possibility is that PI3K is involved in the generation of reactive oxygen species (ROS), which has a negative impact on motility (Luconi *et al.*, 2001).

The increase in motility due to *in vivo* Viagra™ treatment is a novel finding and only speculations can be made regarding the intracellular mechanisms underlying the finding at this stage. Sildenafil citrate induced similar changes in sperm motility parameters as non-selective PDE inhibitors such as pentoxifylline (Calogero *et al.*, 1998; Nassar *et al.*, 1999), caffeine (Cheng & Boettcher, 1981; Leclerc *et al.*, 1996) and IBMX (Leclerc *et al.*, 1996). The intracellular mechanism responsible for the *in vitro* sildenafil citrate-induced cAMP increase in human spermatozoa is also unknown, but it can be hypothesised that sildenafil citrate could act on PDEs other than type 5 (Lefiévre *et al.*, 2000). These observations also suggest that sildenafil citrate probably acts on types 1 and 4 PDEs because cAMP has been implicated in the regulation of sperm motility (Calogero *et al.*, 1998) through activation of PKA (Yanagimachi, 1994).

Both of these motility-enhancing effects need to be investigated further in order to clarify their exact mechanisms of action.

8.1.2 Acrosome reaction

From the results obtained in this study it was evident that:

i) Extracellular signal regulated kinases are directly or indirectly involved in the signal transduction pathway through which the *zona pellucida*-induced acrosome reaction (physiologically relevant exocytotic event) is mediated and not the spontaneous or calcium ionophore induced acrosome reaction.

ii) Phosphatidylinositol 3-kinase increase sperm-*zona* binding without influencing the acrosome reaction.

iii) Inhibition of phosphodiesterase type 5 by sildenafil citrate and therefore possible accumulation of cGMP produce an increase in sperm-oocyte binding. It also suggests that phosphodiesterase type 5 is not strongly involved in the events leading to the human sperm acrosome reaction.

By combining these results with current facts from the literature, a hypothesised schematised representation of the intracellular signal transduction pathways involved in the *zona pellucida*-induced acrosome reaction will be shown in Figure 1 and discussed subsequently.

The ZP3 glycoprotein binds to at least two receptors in the plasma membrane. One receptor is a G_i-coupled receptor that activates phospholipase C (PLC) $_{\beta 1}$. The other receptor is a tyrosine kinase receptor (TKR) coupled to PLC_γ. Binding to the G_i-

coupled receptor would activate adenylate cyclase (AC) leading to the elevation of cAMP and PKA activity. PKA activates a voltage-dependent Ca^{2+} channel in the outer acrosomal membrane, which releases Ca^{2+} from the interior of the acrosome to the cytosol. This relative small rise in $[Ca^{2+}]_i$ could result in the activation of PLC_γ.The products of phosphatidyl-inositol biphosphate (PIP_2) hydrolysis by $PLC_{ß1}$ and PLC_γ, diacylglycerol (DAG) and inositoltrisphosphate (IP_3) lead to PKC translocation to the plasma membrane and its subsequent activation. This increase in $[Ca^{2+}]_i$ can be mimicked by the addition of a calcium ionophore (e.g. A23187), which will also result in the activation of PLC_γ, and PKC activity. PKC opens a voltage-dependent Ca^{2+} channel in the plasma membrane, increasing the $[Ca^{2+}]_i$ even more. PKC activation also results in the activation of ERK, through its ability to phosphorylate and activate an upstream mediator of the ERK cascade, Raf. Raf will activate and phosphorylate the ERK kinase, MEK, which in turn activates ERK. The G_i-coupled receptor and TKR can also activate a Na^+/H^+ exchanger, leading to alkalisation (pH increase) of the cytosol. The increase in $[Ca^{2+}]_i$ and pH will lead to membrane fusion and acrosomal exocytosis.

From the literature it is evident that various crosstalk mechanisms are involved in phosphatidylinositol 3-kinase (PI3K) and RAS/RAF/ERK signalling. It is known that RAS can both activate RAF/ERK as well as PI3K (Sears & Nevins, 2002; Krasilnikov, 2000). PI3K on the other hand can also activate the RAS/RAF/ERK pathway through mitogen signalling (Krasilnikov, 2000). In our experiments we stimulated the AR with solubilized ZP (ZP3), thereby eliciting the AR through both a G_i-protein receptor and ZRK binding which directly activates RAS/RAF/ERK and PI3K through RAS. Inhibition of PI3K by LY294002 thus only inhibited the PI3K pathway and not the

direct RAS/RAF/ERK pathway activated independent of PI3K signalling or activation through PKC. The activation of RAS by ZP binding to ZRK can also stimulate PI3K, thereby enhancing a possible positive feedback on ERK activation (e.g. via PKC) as well as other mediators in the process of AR signalling.

It is well known from the literature that cGMP serve as a signal transducer mediating the AR. When this signalling pathway is activated by using 8-Br-cGMP, effects are only documented at concentrations of higher than 0.5mM with peak activity at 1mM (Rotem et al., 1998; Revelli et al., 2001). This is most likely due to PKA stimulation because cGMP can interact with the regulatory subunit of PKA when used at concentrations higher than 100µM (Lincoln and Cornwell, 1993).

Compared with signalling pathways in many types of somatic cells, the signal transduction pathways of the human acrosome reaction (pellucida glycoprotein) are still very poorly understood (Fisher et al., 1998). One of the chief reasons for this is that the sperm receptor(s) that bind to *zona* protein 3 (ZP3) and physiologically induce the acrosome reaction remain to be conclusively identified (Brewis and Moore, 1997). Indeed, in the human, about ten sperm determinants in spermatozoa have been reported to be involved. The most likely scenario is that several sperm determinants are involved in sperm-*zona* binding and acrosome reaction, functioning in concert as part of a complex, while crosstalk can also occur between different intracellular signalling systems (Kopf *et al.*, 1995; Brewis and Moore, 1997). [In Fisher *et al.*, 1998]

A thorough understanding of signal transduction in human spermatozoa will ultimately yield information regarding the nature of receptors to which these signal transduction pathways are coupled as well as the intracellular effectors that ultimately regulate sperm function. Moreover, an understanding of these regulatory pathways will be essential for the future development of clinical approaches designed to enhance or preclude fertilization.

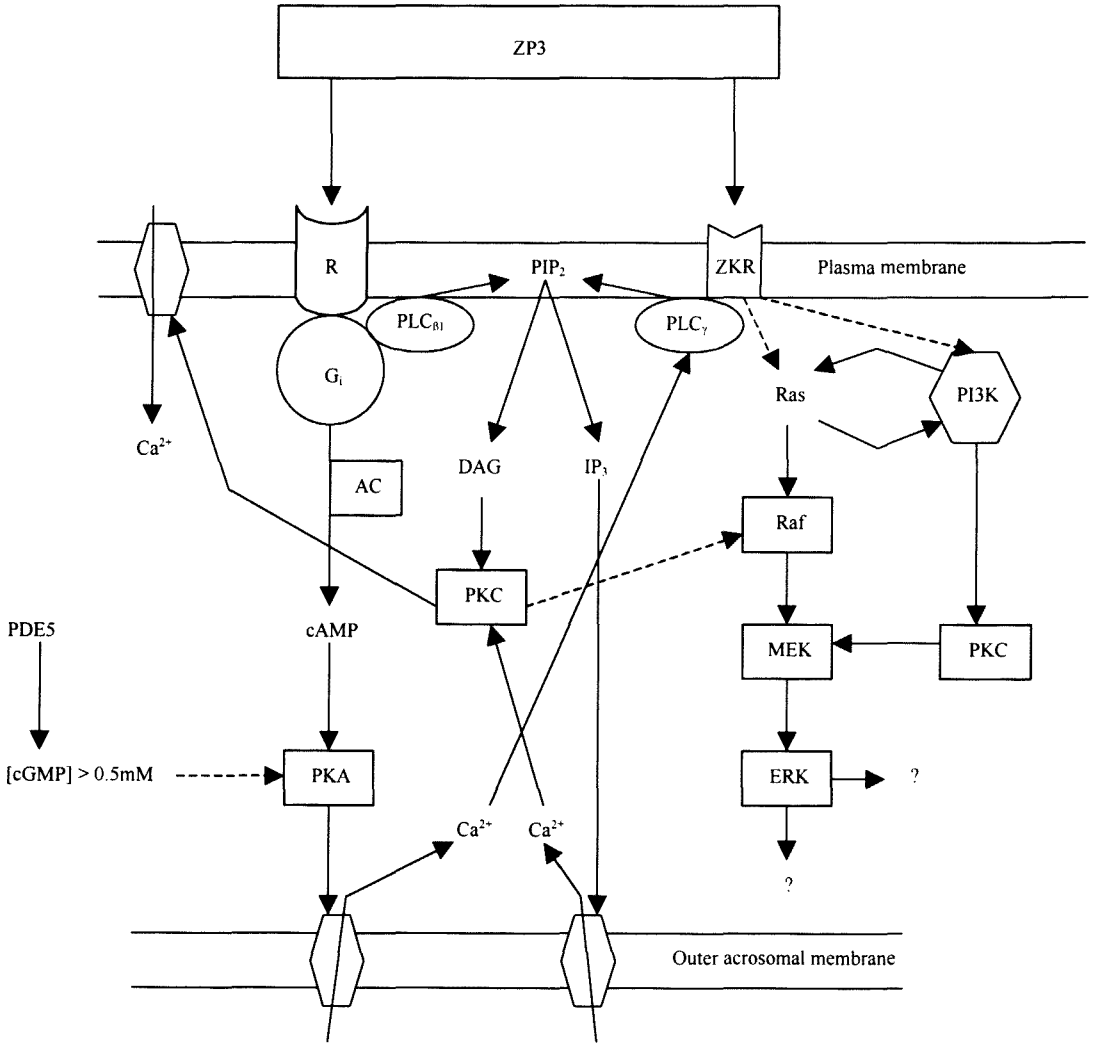

Figure 1: *Hypothesised signal transduction pathways and possible interactions between them. (Dashed lines = hypothesised activation/stimulation)*

8.2 Recommendations

In the field of reproductive biology the emphasis are for various reasons currently shifting away from the female and directed more towards finding solutions for male contraception and male infertility.

Due to the fact that hormonal manipulation (as is the basis of most female contraceptives) as a male contraceptive has so far not been very successful as it can lead to various side effects and even render the male permanently infertile, the need has arisen to look at other fronts. On the other hand, the cure for male infertility factor due to asthenozoospermia often leads to expensive intracytoplasmic sperm injection (ICSI) procedures which is still surrounded by controversy regarding the outcome of the health of the conceptus (Oehninger, 2001; Ola *et al.*, 2001; Simpson & Lamb, 2001). If the possibility exists to increase the motility of spermatozoa without interfering with the rest of the sperms normal physiological processes needed for fertilization, these patients can automatically become candidates for relatively less expensive IVF treatments (Edirisinghe *et al.*, 1995).

With this in mind the following recommendations can be made regarding solutions at the level of intracellular signalling in the spermatozoon:

i) It is recommended that in the quest to preclude fertilization or finding a male contraceptive the possible role of ERK inhibitors and MAPK agonists and antagonists need be further investigated. Our data demonstrate that ERKs are directly or indirectly involved in the acrosome reaction induced by human *zona pellucida*. The results also indicate the importance of intact acrosomes to ensure tight binding to the

ZP, while it is generally accepted that the spermatozoa must be acrosome-reacted to complete penetration of the *zona pellucida*.

ii) Our results imply that PI3K negatively regulates sperm motility and increase sperm-*zona* binding without influencing the acrosome reaction. We subsequently suggest a possible therapeutic role for PI3K inhibitors in the treatment regime for asthenozoospermia. This suggests that LY294002 can be used as a tool to enhance the motility of sperm samples during preparation for IVF from patients with low sperm motility, thereby ultimately opening a new prospective for severe oligoasthenozoospermic males to enter IVF rather than ICSI programs.

iii) The ability of sildenafil citrate to enhance sperm kinematics results and sperm-oocyte binding without affecting the acrosome reaction may be clinically useful. These findings could have important implications in the use of sildenafil citrate in assisted reproductive techniques in order to enhance the fertilizing potential of inherently poor quality sperm with less invasive treatments such as intra-uterine inseminations, thereby avoiding more involved procedures such as IVF or ICSI.

8.3 Future research

The requirement for sperm capacitation as well as the acrosomal status of the spermatozoa travelling through the cumulus oophorus needs to be studied further. Although efforts have been made to establish the characteristics of the proteins present in the sperm surface that acts as the receptor for ZP3 glycoprotein, the exact function for these proteins in the mechanism associated to the ZP3-induced AR has not yet been elucidated. Thus future studies should focus on determining the exact

nature of these receptors, their structure and function. The presence of neuronal receptors (such as GABA-like and glycine receptor) in mammalian spermatozoa with a role in AR, either induced by progesterone or solubilized ZP needs also be investigated (Morales and Llanos, 1996). In addition, the AR, a major regulatory event for mammalian sperm-oocyte interaction leading to fertilization requires further studies to integrate the information regarding the role of the different signalling pathways. These studies may provide the precise sequential role of the intracellular messengers produced after the spermatozoa-ZP interaction has taken place. The nature of the *zona* receptor (ZP2) in the acrosomal-reacted spermatozoa and the acrosomal proteases in the development of the AR and penetration through the ZP need to be clarified. Investigations of these and other related questions should unravel the molecular processes involved in the acrosome reaction.

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"It's not your blue blood, your pedigree or your college degree. It's what you do with your life that counts."

- Millard Fuller -